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CYSTATIN C AND THE RELATION TO CARDIOVASCULAR DISEASE

Studies on the relative importance of genetic and environmental factors

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CYSTATIN C AND THE RELATION TO CARDIOVASCULAR DISEASE
Studies on the relative importance of genetic and environmental factors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Various manifestations of atherosclerotic cardiovascular disease are a common clinical problem affecting millions of people each year and the prevalence increases globally. This strengthens the incentives to improve the tools for primary and secondary prevention. Cystatin C is, besides its role as a marker of renal function, a promising biomarker of vascular damage and vascular disease, which might add value to risk prediction in cardiovascular burdened individuals as well as in persons free of cardiovascular disease. Atherosclerotic cardiovascular diseases are polygenic disorders, and disease development is a function of complex relationships between several environmental factors and multiple genetic variations. The relative impact of genes and environment on the variations in, biomarkers such as cystatin C and creatinine and on their association to cardiovascular disease (CVD), is not well-known and requires further studies. The overall aim of this thesis has been to study the relation between cystatin C and cardiovascular disease, to investigate if it may be used as a biomarker for atherosclerotic disease and if it could lead to earlier identification of patients at particularly high risk. In addition, we investigated the heritability of cystatin C and its relation to heritability of cardiovascular disease. Finally we studied the predictive value of cystatin C for incident CVD when controlled for genetic confounding in twin studies.

Material and methods. This thesis is based on studies in two different study groups. The first consists of elderly men with peripheral arterial disease (PAD) (n103=) and matched controls (n=96) and the second is a population based cohort of elderly Swedish twins of both sexes (n=12313). Initially we performed a cross-sectional study in which we investigated differences in cystatin C-levels between PAD-patients and matched controls. We further studied the predictive value of cystatin C with regards to secondary cardiovascular events in the same group. In the twin study group we investigated the heritability of cystatin C and prevalent CVD using a structured equation model (SEM) followed by a genome-wide complex trait analysis (GCTA). Finally the predictive ability of cystatin C for incident atherosclerotic cardiovascular disease (ASCVD) during a follow-up of 71 month was studied in an adjusted Cox-regression model in twins free from CVD at baseline (n= 11402). Twin pairs discordant for incident ASCVD during follow up were identified and within-pair comparisons regarding cystatin C and creatinine levels were performed.

Results. We observed that cystatin C-levels were higher in PAD patients compared to healthy controls even when corrected for differences in eGFR, IL-6 and CRP. In follow-up we could not establish cystatin C as a predictive marker of incident cardiovascular events in

patients with manifest PAD, however we unexpectedly found a U-shaped relation between tertiles of cystatin C-concentration and outcome. Further we observed a higher heritability of cystatin C compared with previous studies, for which the GCTA analysis provided independent evidence. We also observed a significant genetic correlation between levels of cystatin C and CVD. Lastly we showed that cystatin C was a predictor for incident myocardial infarction (MI) and stroke. The association for stroke was higher than for MI, and that cystatin C remained a predictor for incident stroke after adjustment for genetic confounding.

Conclusion. Cystatin C is associated to atherosclerotic disease. The covariation between cystatin C and CVD in males indicates that cystatin C and CVD share genetic influences. Variation in cystatin C is associated with incident myocardial infarction and stroke independent of traditional risk factors, with a stronger association to stroke. The finding that cystatin C is related to incident stroke in disease-discordant identical twins indicates that individual specific environmental factors are important. One possible explanation is that cystatin C may be a sensitive marker of early hypertensive end organ damage. It could be of value to expand the usage of cystatin C beyond renal medicine and include it as a tool in the arsenal for cardiovascular risk stratification. However, further research is needed.

LIST OF SCIENTIFIC PAPERS

I. **Cystatin C – A marker of peripheral atherosclerotic disease?**
J.Arpegård, J. Östergren, U. de Faire, L-O. Hansson, P. Svensson
Atherosclerosis 199(2008)397-401

II. **Amino-terminal pro-B-type natriuretic peptide and high-sensitivity C reactive protein but not cystatin C predict cardiovascular events in male patients with peripheral artery disease independently of ambulatory pulse pressure**
P.H. Skoglund, J. Arpegård, J. Östergren, P. Svensson
American Journal of Hypertension, 2014;27(3):363-371

III. **Comparison of Heritability of Cystatin C and Creatinine-Based Estimates of Kidney Function and Their Relation to Heritability of Cardiovascular Disease**
J. Arpegård, A. Viktorin, Z.Chang, U. de Faire, P.K. Magnusson, P. Svensson
Journal of the American Heart Association 2015;4:e001467
doi:10.1161/JAHA.114.001467

IV. **Cystatin C predicts incident cardiovascular disease in twins.**
Arpegård J, Magnusson P K E , Chen X, Ridefelt P, Pedersen N L, De Faire U, Svensson P
In submission

CONTENTS

LIST OF ABBREVIATIONS

ABI	Ankle-to-Brachial Systolic Pressure Index
ABP	Ambulatory Blood Pressure
ACC	American College of Cardiology
ACR	Albumin/Creatinine Ratio
AHA	American Heart Association
AMI	Acute Myocardial Infarction
ARVD	Atherosclerotic Renovascular Disease
ASCVD	Atherosclerotic Cardiovascular Disease
CABG	Coronary Artery By-pass Graft
CAD	Coronary Artery Disease
CCr	Creatinine Clearance
CHD	Coronary Heart Disease
CI	Confidence Interval
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration Equation
CV	Cardiovascular
CVD	Cardiovascular Disease
DALY	Disability-adjusted Life Years
DZ	Dizygotic
ESC	European Society of Cardiology
EVA	Early Vascular Ageing
GCTA	Genome-wide Complex Trait Analysis
(e)GFR	Glomerular Filtration Rate (Estimated)
h^2/H^2	Narrow/Broad sense heritability
HR	Hazard Ratio
hs-CRP	High Sensitivity C-reactive Protein
IL-6	Interleukin-6
LDL	Low-density Lipoprotein
MDRD	Modification of Diet in Renal Disease
MI	Myocardial Infarction
MZ	Monozygotic
NT-proBNP	N-terminal pro-B-type Natriuretic Protein
OR	Odds Ratio
PAD	Peripheral Artery Disease
PC	Principal Component
PCI	Percutaneous Coronary Intervention
PP	Pulse Pressure
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Structured Equation Model
SNP	Single Nucleotide Polymorphism
WHO	World Health Organization

1 INTRODUCTION

Atherosclerotic cardiovascular disease is the leading cause of death in both men and women, with an estimated 17.5 million deaths globally in 2012. Further, according to World Health Organization (WHO) estimates, cardiovascular disease as cause of death will increase in both high and low income countries over the next 15 years.[1,2] Within the coming decades it is estimated that the loss of disability-adjusted life years (DALYs) are expected to rise, from 85 million DALYs in 1990 to 150 million DALYs globally in 2020, making it the leading somatic cause of loss of productivity.[3] The main underlying cause of cardiovascular disease, atherosclerosis, and its complications are thus of major importance to public health worldwide.

Risk factors for the development of cardiovascular disease have been thoroughly studied[4] and big efforts and progress have been made in identifying subjects at increased risk. Traditional risk factors such as age, gender, smoking, hypertension, blood lipids and heredity are combined when estimating an individuals' risk of future cardiovascular disease. However, medical endeavors in recent decades have substantially increased survival in patients with cardiovascular disease, creating a growing elderly population with a high prevalence of established cardiovascular disease and cardiovascular risk factors. Traditional risk factors lose some of their predictive ability with increasing age[5] and their predictive ability in established disease is not as well studied in healthy cohorts. In order to identify new markers to improve risk assessment in elderly persons and in persons with known cardiovascular disease it is of importance to identify new predictive markers.

Cystatin C, first and foremost known as a marker of renal function and considered a better marker of glomerular filtration rate (GFR) than serum creatinine, has been suggested as a possible independent biomarker of cardiovascular disease (CVD).[6] Chronic kidney disease (CKD) and cardiovascular disease share common risk factors and often coexist and therefore the relation between cystatin C and CVD is intricate and causal mechanisms are difficult to study. To study the relative importance of how genetic and environmental factors influence variations in cystatin C, how they influence the development of atherosclerosis, and how they influence the association between cystatin C and CVD, may provide new important new knowledge on this relation.

The overall aim of this thesis has been to study the relation between cystatin C and cardiovascular disease, to investigate if it may be used as a biomarker for atherosclerotic

disease, and if it could lead to earlier identification of patients at particularly high risk. In addition, we investigated the heritability of cystatin C and its relation to heritability of cardiovascular disease. Finally we studied the predictive value of cystatin C when controlled for genetic confounding in twin studies.

2 BACKGROUND

2.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a heterogeneous group of disorders related to the heart and blood vessels. They can be further divided into CVD due to atherosclerosis and other CVDs.[7] The first group includes: coronary heart disease (CHD) such as myocardial infarction (MI), cerebrovascular disease, e.g. stroke and peripheral artery disease (PAD), for example intermittent claudication. It is sometimes narrowed down to "hard" atherosclerotic cardiovascular disease (ASCVD), such as in the American College of Cardiology (ACC)/American Heart Association (AHA) guidelines, where it is defined as coronary death or nonfatal myocardial infarction, or fatal or nonfatal stroke.[8] The latter group, which will not be further discussed in this work, includes: rheumatic heart disease, congenital heart disease, cardiomyopathies and arrhythmias.

The onset of atherosclerotic cardiovascular disease starts as early as during fetal life[9,10] and gradually progresses throughout life. By the time the first symptoms occur disease is often quite advanced. Development of atherosclerotic CVD is the result of a vast number of factors that, added together, leads to disease manifestation.[11] These factors are both of a preventable kind such as smoking, unhealthy diet habits and physical inactivity, as well as of non-preventable kind where age, gender, ethnicity, heritability and medical history are the core elements.[12] In individuals with a family-history of CVD the risk for CVD is higher than in individuals without one.[13] This is mainly attributed to the fact that conventional risk factors, due to shared genetics and shared environment,[14,15] are markedly higher in individuals with a family-history of CVD,[16] but does not explain all of the difference.[17] One hypothesis for the unexplained difference in risk for CVD between individuals with a family history of CVD and those without, is that the normal vascular ageing process take on a more rapid course defined as early vascular ageing (EVA).[18] This process may include the morphological changes of arteriosclerosis such as vessel wall thickening and arterial stiffness due to loss of elasticity, molecular alterations such as declining endothelial function, [19,20] and the characteristic changes associated with the atherosclerotic process, such as smooth muscle proliferation and plaque and lesion formation.

2.2 ATHEROSCLEROSIS

Atherosclerosis is a complex chronic inflammatory process engaging the walls of arterial blood vessels[21] developing over many years. The process includes the uptake of low-density lipoproteins (LDL) in the arterial wall, oxidation of LDL, which generates an

inflammatory reaction with leukocyte infiltration into the vessel wall and uptake of LDL into macrophages, with the formation of foam cells.[22] Smooth muscle cells migrate within the vessel wall, forming a fibrous coat around the plaque, but this coat can be weakened which in turn may lead to plaque rupture and thrombosis. This ultimately lead to complications such as acute myocardial infarction (AMI) and stroke, which together amounts to roughly eighty percent of all cardiovascular (CV) death in both sexes worldwide.[7]

One of the most prominent clinical manifestations of atherosclerosis is peripheral arterial disease. The main morphological feature of PAD is arterial stiffening,[23] arteriosclerosis, which in turn leads to arterial remodeling, vessel wall thickening, atheroma buildup and lastly atherosclerotic plaque rupture.[24,25] Symptoms range from intermittent claudication, with painful cramping in the lower extremities during physical activity to ischemic ulcers and critical limb ischemia, which ultimately may result in amputation of parts of the extremity.[26] Peripheral artery disease is common, but since it is often asymptomatic the prevalence is hard to estimate. A population based study in 5000 individuals reported a prevalence of >18% for persons 60-90 years in Sweden.[27] It is also associated with a high frequency of concomitant coronary artery disease (CAD) which is often silent [28] and patients are also at increased risk for other cardiovascular events.[29,30]

2.2.1 Remodeling of the extracellular matrix

One important component in the atherosclerotic process is the remodeling of the extracellular matrix in the adjacent vessel wall around the plaque.[31] This process is related to changes in the vessel wall called arteriosclerosis, that occur in normal vascular ageing, as a consequence of adaptive mechanisms to preserve normal conditions of blood-flow and wall tension.[32]

Proteolytic enzymes have the ability to degrade components of the extracellular matrix, such as elastin and collagen, by elastinolysis and collagenolysis. These can be seen as compensatory mechanisms and they are important in the progression of atherosclerosis and aneurysm development.[33,34] Matrix metalloproteinases are examples of such proteolytic enzymes that have received considerable attention.[35,36] More recently it has been shown that also cathepsin-family cysteine proteases are important proteins in the elastinolytic process. This group includes several proteins, such as Cathepsin S and K, which can be measured in serum and therefore have been proposed as possible biomarkers for atherosclerotic disease.[37-39] A natural inhibitor of the cathepsin cysteine proteases is cystatin C.[40]

2.3 CHRONIC KIDNEY DISEASE IN RELATION TO CVD

Decreased glomerular filtration rate(GFR) is a known risk factor for the development of cardiovascular disease in subjects with mildly reduced kidney function as well as in individuals with established chronic kidney disease,[41-43] and merely microalbuminuria is associated with increased risk for coronary events independently.[44] Therefore, early identification of chronic kidney disease on the basis of proteinuria alone or together with reduced glomerular filtration rate is important in order to permit early intervention.[45] Individuals with chronic kidney disease have a higher risk of dying from cardiovascular disease than from kidney failure.[46] Although much of the risk of CKD is due to its association with other traditional CVD risk factors, such as more severe hypertension or dyslipidemia, several studies have noted that the traditional Framingham risk-factors for CVD do not sufficiently capture the CVD risk in CKD patients.[47] If this is an effect due to non-traditional risk factors or an altered effect of traditional risk factors in CKD patients, or if it is the presence of CKD in itself that is an independent risk factor for CVD outcomes, is not yet fully understood.[48]

2.3.1 Creatinine

The gold standard for measuring renal function is by measuring the clearance rate of injected substances such as inulin or iohexol.[49] These are both precise, but invasive, time consuming and expensive methods and therefore of limited clinical use.[50] In the mid 1930's the clearance of endogenous creatinine, a 113D amino acid,[51] was proposed to reflect the glomerular filtration rate.[52] Ever since, it has been a favored analysis, both in urine and serum, for approximating kidney function in general and glomerular filtration rate in particular. However glomerular filtration rate is merely one of several factors that determines creatinine concentration.[50]

The generation of serum creatinine is primarily the result of non-enzymatic dehydration of muscle creatine[53] and the diary intake of creatinine from heated meat.[54] Thus, muscle-wasting conditions such as glucocorticoid medication[55] and diseases such as Duchenne's muscle dystrophy and myasthenia gravis[56] will also affect serum creatinine, as will sex,[57] cachexia and natural ageing.[58]

The renal handling of creatinine is a complex matter since creatinine does fulfill some, but not all, requirements for a perfect filtration marker. It is freely filtered, not protein bound, not metabolized by the kidney and it's physiologically inert.[50] However it is partly secreted by

the proximal tubuli, making creatinine clearance (CCr) based GFR uncertain in some cases.[59] There are also indications that decreased urinary output, such as in patients with decompensated heart failure, may cause tubular reabsorption of creatinine.[60,61] Some drugs, for example trimethoprim, will inhibit creatinine secretion and thus reduce creatinine clearance, which will lead to an increased creatinine level without affecting the GFR.[62] There is also an extrarenal creatinine elimination pathway in the gastrointestinal tract mainly seen in patients with heavily reduced GFR, whereby creatinine from intestinal secretions is degraded by bacteria of the gut.[63]

2.3.2 Estimation of kidney function from endogenous biomarkers

The inaccuracy of creatinine as an endogenous marker of GFR has led to the development of several formulae to circumvent these shortcomings. These estimations often include, age, sex, race and body size in addition to the serum biomarker.[64] One of the earliest formulas developed to estimate GFR from creatinine was the Cockcroft-Gault formula developed in the early 1970's with data from 250 men.[65] After some corrections, it was later also validated for use in women.[66] It has been observed that this formula systematically overestimates GFR, particularly in individuals with GFR <60 mL/min/1.73m^2.[67]

In the late 1990's the Modification of Diet in Renal Disease Study (MDRD) formulated a new creatinine based equation.[68] This formula has been found to perform equal or better than the Cockcroft-Gault formula in most populations[64] but it has been observed that it is imprecise in patients with normal or only mildly reduced kidney function where it underestimates GFR.[69]

The Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI) was introduced in 2009 and uses the same variables as MDRD but it has proven to be more correct, especially in subjects with GFR >60ml/min/1.73m^2.[70] Further, the CKD-EPI equation is the only formula that is also developed for standardized cystatin C,[71] and using a combination of creatinine and cystatin C in the equation has proved to be the most accurate way to estimate GFR across the whole range of GFR as well as in different subgroups.[72] Lastly albumin/creatinine-ratio (ACR) is a way to detect microalbuminuria, which may be an early sign of renal disease before it is detectable as a decrease in GFR.[73]

2.4 CYSTATIN C

Cystatin C is a low molecular weight basic protein (13,343-59 Da),[74] first described in 1961 as γ-trace or post-γ-globulin when it was isolated in normal cerebrospinal fluid and urine from patients with renal failure.[75,76] Later its presence has also been shown in serum, saliva, seminal and synovial fluid.[77] In the early nineteen eighties, it was recognized as a 120 amino acid residues polypeptide, functioning as a cysteine protease inhibitor, located on chromosome 20 and renamed cystatin C.[78-80] The cystatin C gene, called CST3, is of the housekeeping type, meaning that it is required for the maintenance of basic cellular function.[79,81] It is expressed in all nuclei bearing cells, which produces and secretes cystatin C at a fairly constant rate.[80] Cystatin C belongs to Family 2 of the cystatin superfamily. This protein family consists of cystatins C, D, E/M, F, G, S, SA and SB which mainly exert extra- and transcellular effects.[82] The cystatins have several characteristics in common, with the ability to inhibit almost all papain-like (family C1) cysteine endopeptidases being the most significant, and each cystatin molecule have a single reactive site for each peptidase it inhibits.[83]

2.4.1.1 Cystatin C and relation to kidney function

Cystatin C has proven to be almost freely filtered in the renal glomeruli and fully metabolized after tubular reabsorption.[84,85] Normal plasma levels for cystatin C in adult healthy individuals ranges between approximately 0.5-1.2 mg/L but rises reciprocally with the reduction of glomerular filtration rate.[85,86] Several studies have indicated that cystatin C is more precise than creatinine in estimating glomerular filtration rate,[87,88] especially in older populations, [89] and that it improves CKD detection (defined as estimated GFR <60ml/min/1.73m^2). Cystatin C may thus be better at predicting end-stage renal disease and all-cause mortality.[72,90] In contrast to creatinine-based formulas such as MDRD and Cockcroft-Gault, cystatin C-based GFR estimations do not need to take factors such as age, body mass and gender into account.[91-93] Therefore the use of cystatin C as a marker of renal function has increased in clinical practice, although there are a few certain situations where cystatin C's reliability as a marker of kidney function may be limited, such as in patients with thyroid disease, malignancy and during corticosteroid use. [94]

2.4.1.2 Cystatin C and Cardiovascular Disease

Several epidemiological studies have suggested that cystatin C, besides being a marker of GFR, is associated with numerous different clinical outcomes such as heart failure, risk for infection and all-cause mortality.[95-97] Besides this, it has been proposed that cystatin C could be used as an independent biomarker for cardiovascular disease.[6,97] One possible rationale for this independent association is that cystatin C is an important natural inhibitor of cysteine proteases such as the cathepsins B, H, K, L and S, which has a central role in the remodeling elastinolysis of the vessel wall that is an important part of the atherosclerotic process.[31,98] There is also some evidence that cystatin C is associated with early stage arteriosclerosis, defined as increased arterial stiffness measured by cardio-ankle vascular index (CAVI),[99] in subjects with estimated glomerular filtration rate (eGFR) >60mL/min.[100]

Findings from previous population-based studies have shown that cystatin C is superior to creatinine for prediction of incident ASCVD.[101-103] Whether this is due to unique properties of cystatin C that are independently associated to vascular remodeling, and maybe even causally involved in the development of ASCVD, has been debated.[82] Thus it is plausible that it primarily is a reflection of cystatin C being a more sensitive marker of early GFR-reduction.[104] In this regard cystatin C might be a marker of early vascular ageing, and as such detect subclinical manifestation of features such as small vessel degeneration, left ventricular heart load, arterial calcification, matrix remodeling and intima alterations.[105,106]

2.5 CARDIOVASCULAR RISK FACTORS

Numerous factors have been identified that contribute to a risk profile prone to develop cardiovascular disease. Those of greatest importance for the development of myocardial infarction in both sexes, at all ages, in all regions worldwide are: smoking, abnormal lipids, history of hypertension or diabetes, abdominal obesity and psychosocial factors. Protective factors are: daily consumption of fruits and vegetables, moderate alcohol consumption and regular physical activity.[4] In a global perspective hypertension, smoking, physical inactivity, unhealthy diets and harmful use of alcohol are the most important modifiable factors.[7]

Both genetic- and environmental factors in a complex interplay contribute to the development of a risk profile. A distinct example is hypercholesterolemia, which is characterized by elevated levels of LDL-cholesterol in plasma that may be attributed both to dietary intake as well as a genetic predisposition in the form of familial hypercholesterolemia with hetero or homozygotic pathogenic variants in one of three genes (*LDLR, APOB, PCSK9*).[107] There is also evidence that other single nucleotide polymers (SNPs) associated with levels of LDL are

also independently associated with a risk of first myocardial infarction, ischemic stroke, or death from coronary heart disease.[108,109]

2.5.1 Risk scoring and guidelines

The regional scientific societies, such as the European Society of Cardiology (ESC) and the American Heart Association (AHA) regularly publish guidelines to provide health care professionals with up to date, evidence based knowledge and recommendations regarding the management and prevention of cardiovascular disease.[110,111] In order to aid physicians in estimating cardiovascular risk for clinically healthy individuals, these guidelines include risk scoring systems such as the QRISK, the JSB3, the Reynolds risk scores for men and women and most recently the ACC/AHA hard ASCVD risk calculator.[8,112-115]

Two of the most frequently used risk scoring systems are the Framingham Risk Score, initially presented in 1998,[116] and the European SCORE-system from 2003.[117] The Framingham Risk Score was revised into its current form, Framingham/ATPIII in 2002,[118] and a version with a broadened endpoint including stroke, heart failure, coronary artery disease and peripheral artery disease named Framingham General Cardiovascular Risk Score was designed in 2008.[119]

Both Framingham and SCORE provide a 10-year assessment of cardiovascular risk in previously healthy individuals, SCORE for first fatal atherosclerotic event and Framingham for general cardiovascular disease risk.[117,119] Both these scoring systems include age, sex, total cholesterol, systolic blood pressure, high-density lipoprotein and smoking. Framingham also includes blood pressure treatment and diabetes, whereas SCORE on the other hand includes high or low risk region of Europe as a risk factor.

A general disadvantage of all current risk prediction systems, which are mainly based on the Framingham study,[120] is that they may vary in accuracy between different ethnic groups, cultures and ages.[121,122] Further, none of the scoring systems available today are designed for subjects already burdened by cardiovascular disease and coronary heart disease equivalents such as PAD, cerebrovascular disease, abdominal aorta aneurysms and diabetes mellitus.[123] Patients with any of these conditions present are placed in the high-risk group but current guidelines do not offer a further risk stratification within these populations.

The presence of chronic kidney disease or decreased renal function is only to a limited extent considered in current guidelines and scoring systems. Despite the fact that individuals with CKD are at high risk for cardiovascular disease,[124] and that glomerular

filtration rate and albuminuria are consistently associated with elevated cardiovascular risk in many different populations,[125] only JSB3 and QRISK incorporate CKD in the equations. In the ESC guidelines on cardiovascular disease from 2012 an estimated GFR < 60 mL/min per 1.73 m^2 is viewed to confer high or very high risk, whereas urinary ACR of 30–300 mg/g is only viewed to confer risk in patients with diabetes and albumin/creatinine ratio above 300 mg/g is not considered at all.[111] The ACC/AHA guideline considers the contribution of both CKD and albuminuria to be uncertain and thus issues no recommendations for or against.[8] None of the scoring systems recognizes the measurement of estimated glomerular filtration rate or ACR as risk factors.

Most cardiovascular events will occur in individuals at moderate risk. Thus, from an overall perspective it makes sense to identify subjects at moderate risk who are in need of preventive measures on the basis of their global risk score, derived from all CVD risk markers. However, it is individuals within high-risk groups that will have the greatest benefit of reducing risk factors[126] due to a higher reduction of their absolute risk.

Therefore, in recent years increased emphasis is being placed on reducing the absolute risk for the individual instead of reducing the relative risk on group level.[127] This has led to an increasing interest in different blood-biomarkers as they might add to the absolute individual risk in elderly populations with cardiovascular disease, where the relevance of traditional risk factors decreases.[5] As of yet though, the only blood-biomarker embraced by current guidelines is high sensitive C-Reactive Protein (hs-CRP) and its relevance in enhancement of risk improvement is disputed.[128] Consequently, finding new ways to more accurately predict cardiovascular risk, including the development of new biomarkers, is of importance.

2.6 COMPLEX DISORDERS

Genetically, atherosclerotic disease belongs to the polygenic, or complex, disorders. This means that the development of a disease (phenotype) is a function of a complex relationship between several environmental factors and multiple genetic variations.[129] Unlike Mendelian disorders, such as Huntington's disease, cystic fibrosis or phenylketonuria, which are caused by alterations in one gene alone, complex disorders does not obey the Mendelian single-gene dominant or recessive patterns of inheritance.[130] Although the genes associated with complex disorders are inherited in the same way, they only make up part of the disease pathway. This means that a genetic susceptibility for developing a complex disease may not be enough, interaction at the molecular level between genetic products and by-products of environmental impact is also needed for the disease phenotype to occur.[131] Further, the

genetic prerequisites for complex disorders are almost invariably due to alterations at more than one genetic locus, and interaction between many different loci (epistasis) [132] may contribute to disease development. This has been confirmed in large scale association analyses that have identified over 45 genetic loci in individuals of European and Asian descent associated with cardiovascular disease, whereof 1/3 of loci is also related to the traditional risk factors plasma lipids or hypertension.[133-135]

2.7 QUANTITATIVE GENETICS

Quantitative genetics is a method to study if observed differences among individuals regarding complex traits are due to genetic or environmental differences, or both. The most commonly used methods in these types of studies are twin and adoption studies. Many heredity studies nowadays also incorporate a molecular genetic method called genome-wide complex trait analysis (GCTA) in order to strengthen the findings from the classical heritability model.[136]

2.7.1 Heritability and environment

The proportion of the variation of a phenotype that is due to heritable (genetic) differences between individuals within a study population is referred to as heritability. Heritability can be measured in two different ways, broad-sense (H^2) and narrow-sense (h^2). The broad-sense heritability measures the ultimate ability to predict a phenotype from a genotype as it measures the full contribution of genes to the phenotype. This can be broken down further into contribution from individual (additive) alleles, contributions due to homologous alleles at a specific locus (dominance) and combinations of non-homologous loci (epistasis). The narrow sense heritability captures the additive contribution of genes to the trait, which is the same as the maximum variance that can be explained by a linear combination of the allele counts.[137,138]

2.7.1.1 Structural equation model (SEM)

To estimate heritability for the chosen phenotypes the structural equation model (SEM) fitting-method decomposes phenotypes into the influence of an additive genetic factor (A), a common environmental factor (C) or dominance genetic factor (D), and a unique environmental factor (E).[136,139] The broad-sense heritability is then estimated as either the proportion of variance explained by A plus D, if the intra-class correlation in monozygotic twins is larger than twice of the correlation in dizygotic twins (ADE-model), or if the intra-class correlation in monozygotic is less than twice the correlation in dizygotic (ACE-model) only by A.

2.7.1.2 Bivariate heritability

In certain circumstances it may be of interest to investigate if the covariance of two, or more, phenotypes is due to genetic or environmental factors, for example serum level of cystatin C and glomerular filtration rate. This can be accomplished by using bi-, or multivariate quantitative genetics.[140] By utilizing cross-covariance between family members it is possible to study whether trait A in a family member is associated with trait B in another family member. A genetic correlation of 1.0 implies that all additive genetic factors that influence trait A also influence trait B. In analogy, a shared environmental correlation of 1.0 suggests that environmental influences that make family members similar with regards to trait A also influences family members similarity with regards to trait B to the same extent. Consequently, a high genetic correlation in bivariate analysis implies that a gene that influences one of the investigated traits is also likely to influence the other investigated trait. Another important factor regarding bivariate heritability is that genetic correlations are independent of univariate heritabilities. Hence trait A and trait B may both have low heritabilities but still have high genetic correlations, implying that even though the genetic effect on the phenotypes is small the genes involved most certainly influences both traits.[136]

2.7.1.3 Gene-environment interplay

The interplay between genes and environment is roughly divided into gene-environment correlation and gene-environment interaction. The former is subsequently divided into the passive-, evocative- or active type where passive means that children receive genotypes that are correlated with family environment, evocative implies that individuals are reacted to on the basis of their genetic prerequisites and active assumes that individuals create or seek out environments that correlates with their genetic makeup.[141] Gene environment-interaction is usually defined as the effect the environment has on a phenotype on the basis of the underlying genotype. A consequence of this is that individuals with predisposing genetics may be especially vulnerable to the influence of certain environments.[142] A theoretical example is exposure to ultra violet radiation from the sun, which could lead to unfavorable mutations in a susceptible person which in turn eventually leads to development of malignant melanomas.

2.7.2 Twin-study methodology

The strength of the classic twin study model is due to the fact that it compares monozygotic twins, who stems from the same egg and thus share all alleles, with dizygotic twins who are developed from two eggs and on average shares 50% of their genome.[143] These genomic

prerequisites, paired with a number of assumptions that need to be accepted, make up the basis of the twin study design which makes it possible to study the effects of genetic and environmental variance on a specific phenotype.

In 1875 Sir Francis Galton published the results of his twin studies. Although his studies merely focused on the impact of environment on twins and thus did not compare resemblance between monozygotic (MZ) and dizygotic (DZ) twins, his studies are considered the first of its kind. In these publications, he argues that nature, i.e. genetics, is a more important factor than the environment in the development and behavior of twins. He also argued that twins originate from the same egg, despite having any heavy evidence for this claim.

A breakthrough regarding the logic of twin study methodology were made through studies conducted in the early 1920s by psychologist Curtis Merriman and dermatologist Herman Werner Siemens.[144] Siemens counted the number of birthmarks in MZ-twins and compared the result with the number in DZ-twins. He then found the correlation of the number of moles in MZ to be 0.4 compared with 0.2 in DZ, who only share half of their genome. This conducted him to formulate the hypothesis that all heritable properties will exhibit greater similarity in monozygotic than in dizygotic twins.[145] Thus, by combining classical correlation methods with the study of twins the modern twin study was born.

As a result of the fact that they are born at the same time and place, twins share many environmental aspects from the intra-uterine environment through parenting style to education and socioeconomic factors. In theory this means that any difference in the presence of a specific genetic trait between twins in a MZ pair (discordance) is due to unique environmental factors that impact the trait in one but not the other. This can be true if the following assumptions are made:

- the assumption of equal environment, assuming that both MZ- and DZ-twins share common environment to the same extent.
- the assumption of random mating, i e no inbreeding or assortative mating.
- the assumption of minimal gene-environment interaction/correlation, meaning that exposure to environmental conditions is dependent on an individual's genotype.
- the assumption that there is no difference regarding the investigated traits between twins and the general population.[146]

In reality it is possible that one or more of these assumptions do not hold true, which will have implications for the result of the twin study, for example the equal environment assumption has been largely debated. A violation of this assumption would be if MZ twins share more trait relevant environment than do DZ, since this would lead to an increased MZ correlation relative to DZ correlation, which in turn can result in an overestimation of the genetic effect and an underestimation of the shared environmental effect.[146] In favor of the equal environment assumption is the fact that MZ twins that were brought up apart display similar correlations compared to MZ twins who were brought up together.[147]

By calculating the difference in correlation for a particular phenotype between MZ and DZ twins, it is possible to calculate the heritability (h2) of this phenotype.[148] Heritability, or heredity, in the example above is therefore ((0.4 - 0.2) x 2) = 40%. Often, it is of interest to calculate to what extent a common environment affects the expression of a particular property or disease. This can be done by calculating the difference between total correlation within the MZ twin group and the heritability (h2). In a Swedish study of antisocial behavior in girls a correlation of 0.82 in MZ and 0.45 in DZ was calculated, which gives an h2 of 74%.[149] On the basis of this it's possible to consequently estimate a common environmental factor of 8% (0.82 - 0.74). The difference between perfect correlation of 100% and the measured correlation in MZ, in this case 1.0 - 0.82 = 0.18, is said to arise from individual specific environmental factors.

2.7.3 Genome-wide Complex Trait Analysis (GCTA)

Genome-wide complex trait analysis is a recently developed method whereby the proportion of variance of a complex trait that is explained by common genetic variation is estimated using single nucleotide polymorphism- markers(SNPs).[150] Contrary to the twin design, GCTA does not rely on familial resemblance. Instead it uses thousands of individuals to find genetic similarity across hundreds of thousands of SNPs. The power of the method lies in the fact that it can extract tiny signals of genetic similarity from the hundreds of thousands of SNPs investigated by comparing them for each pair of individuals in a matrix of thousands of unrelated individuals.[136] One of the major advantages of the method is that the heritability estimated comes directly from measured DNA differences between the unrelated individuals, thus making it a between-family analysis method as opposed to a twin structured equation model (SEM) that is within family. Thus, the twin-SEM and GCTA is not directly comparable since GCTA is restricted to additive contribution from common variants while twin-SEM can capture both additive and dominance from both common and rare variants.

GCTA can also be extended to bivariate heritability analysis,[151] and in contrast with univariate analysis bivariate estimates of genetic correlation from GCTA are similar to estimates from twin studies, as GCTA estimates of genetic correlations are unbiased.[152]

2.8 BACKGROUND SUMMARY

Various manifestations of atherosclerotic cardiovascular disease are a common clinical problem affecting millions of people each year.[1,3] The fact that both prevalence and incidence increases globally[2] strengthens the incentives to improve the tools for both primary and secondary prevention. Identifying new potential ways to use established biomarkers may allow for earlier and more precise identification of high risk individuals and take preventive measures before patients suffer complications. Increased knowledge of the above relations may also eventually provide new therapeutic opportunities.

Cystatin C is a promising biomarker of vascular damage and vascular disease,[6,97] that might add value to risk prediction in cardiovascular burdened individuals as well as in persons free of cardiovascular disease. Patients with peripheral arterial disease are an interesting group to study with regards to cystatin C since manifest disease features both arteriosclerosis and atherosclerosis and thus is well suited for studies of possible connections between these conditions and cystatin C. Further they are a, often undertreated, group with high cardiovascular risk where coronary artery disease is the major cause of death[6,29,153] and they are in need of improved tools for risk prediction.

Cystatin C serves as an endogenous marker of glomerular filtration rate, possibly less biased than creatinine.[92] Chronic kidney disease is a known risk factor of CVD.[44] Nevertheless, chronic kidney disease and cardiovascular disease share common risk factors and often coexist. Therefore the relation between cystatin C and CVD is intricate and causal mechanisms are difficult to study.

Atherosclerotic cardiovascular diseases are polygenic disorders and disease development is a function of complex relationships between several environmental factors and multiple genetic variations.[129] To study the relative importance of how genetic and environmental factors influence variations in cystatin C, how they influence the development of atherosclerosis, and how they influence the association between cystatin C and CVD, may provide new important new knowledge on their relation. The main purpose of this thesis was to further investigate these gaps in knowledge.

3 AIMS OF THE THESIS

The overall aim of this thesis is to deepen the understanding about the relation between cystatin C and cardiovascular disease. We wanted to investigate whether cystatin C could be used as a biochemical marker for atherosclerotic cardiovascular disease *(Study I*) and if it had prognostic value in cardiovascular patients *(Study II)*. In addition, we intended to estimate the heritability of cystatin C *(Study III)* and to investigate which role genetics and environment have on the relationship between cystatin C and prevalent *(Study III)* as well as incident *(Study IV)* cardiovascular disease.

Specific objectives of the respective sub–studies:

3.1 STUDY I

- To investigate if cystatin C, independent of its function as a marker of glomerular filtration, could be an independent marker of peripheral arterial disease.

3.2 STUDY II

-To examine the predictive value of cystatin C in patients with PAD. Secondary aims were to study whether predictive models including cystatin C resulted in better discrimination and correctly reclassified patients in comparison with a model containing other significant risk factors previously identified in this cohort.

3.3 STUDY III

- To estimate the relative importance of genes for the phenotypic variability of cystatin C and creatinine levels, in a well-powered twin study. A secondary aim was to study the relation of heritability of cystatin C and creatinine to the heritability of cardiovascular disease.

3.4 STUDY IV

- To study if variations in levels of cystatin C, predicts incident atherosclerotic cardiovascular disease when controlled for traditional risk factors and genetic factors. A secondary aim was to study the association of cystatin C to incident MI and stroke respectively.

4 SUBJECTS AND METHODS

4.1 SUBJECTS

4.1.1 Study I & II

Study participants were consecutively recruited from patients referred for symptoms of intermittent claudication to the vascular clinics of Karolinska and S:t Göran hospitals, Stockholm, Sweden, between 1998 and 2001. Inclusion criteria were male sex, age >45, a history of intermittent claudication (leg pain at exercise with prompt relief at rest and not explained by another condition), and an ankle-to-brachial systolic pressure index (ABI) <0.9 by Doppler ultrasonography at rest based on initial study examination. Twenty-seven patients had a history of previous peripheral vascular surgery. In these patients, a higher baseline ABI than <0.9 at the time of the study investigation was accepted. Patients with rest pain, previous amputation, or reasons for a reduced walking performance other than intermittent claudication, diabetes mellitus type 1, and atrial fibrillation were excluded. A history of ischemic heart disease was not an exclusion criterion, and patients were included irrespective of presence of ischemic heart disease. To confirm the intermittent claudication diagnosis all patients and control subjects performed a standardized exercise treadmill test. Referred patients that met the inclusion criteria were asked for informed consent to participate in the study.

A total of 103 respectively 99 patients with intermittent claudication met the inclusion criteria for participation in study I and II. In one patient, ambulatory blood pressure (ABP) monitoring was not performed since office systolic blood pressure (SBP) was repeatedly >210 mm Hg, leaving 98 patients for the final analysis in study II. No alteration of medication was done before the investigations.

Potential control subjects matched for sex and age were consecutively drawn from the population registry of Stockholm County. Eligible subjects were invited to the clinic for a screening visit, at which time a medical history, blood pressure, ABI, and a 12-lead electrocardiogram were obtained. Subjects who did not have a history of ischemic heart disease, stroke, or PAD and with an ABI >0.9 were selected as control subjects. The same exclusion criteria applied for patients and controls with regard to type-1 diabetes and atrial fibrillation. A total of 96 control subjects were sampled in study I. In study II 92 control subjects were sampled, 90 of who performed ambulatory blood pressure monitoring. All control subjects provided informed consent.

4.1.2 Study III & IV

Study participants were all obtained from the TwinGene project. TwinGene is a Swedish population based cohort of twins born between 1911 and 1958, contacted and enrolled for testing between the years 2004-2008.[154] All eligible participants had previously participated in a computer assisted telephone interview called SALT (Screening Across The Life Span Twin Study).[155] Further, both twins within the pairs had to be alive and provide their informed consent for study participation. The zygosity of the twins was based on self-reported childhood resemblance, or by DNA-markers (54% of the study sample). According to a recent independent test of the validity of similarity-based zygosity assignments among the adults in the TwinGene study there is a dizygotic to monozygotic error rate of 2.56%, corresponding to an accuracy of 97.4% (95% CI: 96.6–98.2%) .[154] Participants who had previously donated DNA for studies in the Swedish Twin Registry (STR) and participants who had declined participation in further studies or had a record of hepatitis were excluded. In total 12645 individuals donated blood to the project and for these studies serum aliquots from a total of 12570 subjects were withdrawn. Of these 257 were excluded due to bad or missing sample, non-sufficient sample volume, hemolysis, lipemia or missing donor ID leaving a total of 12313 individuals for analysis in study III. Further an additional 911 subjects with prevalent cardiovascular disease on enrollment were excluded in study IV, leaving a total of 11402 individuals for the final analysis.

4.2 METHODS

4.2.1 General assessments

4.2.1.1 Study I & II

Height was measured, without shoes, to the nearest centimeter, weight, without shoes and overcoat, to the nearest kilogram and body mass index (BMI) was calculated (kg/m2). Waist and hip circumference was measured in cm and waist/hip ratio calculated. Waist circumference was measured directly on the body surface midway between the lower rib margin and iliac crest. Hip circumference was measured over light clothing at the widest girth of the hip. Ambulatory blood pressure values were obtained using a non-invasive oscillometric system (Spacelabs 90207, Spacelabs Inc., Redmond, WA, USA). Blood pressure and heart rate were recorded automatically every 15 min for a 24-h period. Office BP was recorded in both arms by an experienced nurse using a mercury sphygmomanometer with the subject in the supine position after 5 minutes of rest. The mean of two consecutive readings was calculated. If there was a difference in systolic or diastolic blood pressure between the arms of >10 mm Hg, the arm with the highest reading was used when defining

office blood pressure; otherwise the non-dominant arm was used.[156] The same arm was used for office and ambulatory blood pressure measurements.

4.2.1.2 Study III & IV

Study participants were asked to make an appointment at their local healthcare facility Monday to Thursday for a morning visit. The subjects' height, weight, hip, and waist circumference was recorded without shoes and in light clothing. Participants were asked to rest for 5 minutes, thereafter systolic and diastolic blood pressure was measured with the subject in upright sitting position. A second blood pressure was taken after 1 minute.

Through the SALT computer assisted telephone interviews [155] data regarding birth order and weight, similarity with sibling, contact with twin partner, common illnesses, prescription and nonprescription medication use, consumption of alcohol, tobacco and caffeine was collected by trained interviewers with adequate medical background.

4.2.2 Laboratory examinations

4.2.2.1 Study I & II

Fasting venous blood samples were drawn and analyzed for creatinine [157]*(Study I & II)* and HbA1c *(Study I).* Creatinine clearance was calculated according to Cockcroft's formula[65] *(Study I)*. GFR was calculated according to MDRD from creatinine age, race and gender *(Study I & II)*.[68,158] The distribution of the estimated GFR was divided into four categories (less than 45.0, 45.0–59.9, 60.0–74.9, and at least 75.0 mL/min/1.73m2) according to the guidelines of the National Kidney Foundation *(Study I)*.[73]Additional blood samples were collected for the determination of serum IL-6 *(Study I)*, cystatin C and high sensitive CRP (hsCRP) *(Study I & II)*. Cystatin C and hsCRP were quantitated according to the instructions of the manufacturer using particle-enhanced immunonephelometric assays with kits and instrument (BN II analyzer) from Dade Behring GmBH, Marburg, Germany. [159] The total coefficient of variation for cystatin C at 1.2 mg/L was < 3.5%. Total coefficient of variation for hsCRP = 3.9% at concentrations below 10 mg/L. IL-6 was measured using a high sensitive ELSA kit according to the instructions of the manufacturer (HS600, R&D Systems, Minneapolis, MN, USA). The lower detection limit was 0.1 pg/mL *(Study I & II)*. The total coefficient variation for IL-6 was <7%. HbA1c was determined using a chromatographic (FPLC, Pharmacia BTG) method with a reference interval <5.2% and a total CV< 3% *(Study I)*. Fasting venous blood samples for NT-proBNP determination were collected in ethylenediamine-tetraacetic acid-containing tubes. The samples were then centrifuged, and plasma was stored frozen in aliquots at −70 °C within 30 minutes. Plasma NT-proBNP

concentration was measured using a commercial test kit and instrument (ELECSYS 2010; Roche Diagnostics, Basel, Switzerland), with a reported coefficient of variation of 3.3% *(Study II)*.[160]

4.2.2.2 Study III & IV

Participants were instructed to fast from 8 PM on the night before the blood sampling. A sample volume of totally 50 mL of venous blood was drawn from each participant. Tubes with serum and whole blood for clinical chemistry analyses and DNA extraction were sent by overnight mail to KI Biobank. Serum samples were aliquoted by Tecan-robot into 1 mL fractions and placed in 1.8 mL cryotubes that were stored in liquid nitrogen tanks at the KI Biobank. Serum aliquots from participating subjects were then withdrawn, thawed and directly shipped off to laboratory for clinical blood analysis. Clinical blood assessments were performed at the Department of Clinical Chemistry and Pharmacology, University Hospital, Uppsala, Sweden. Serum samples were analyzed on Abbott Architect ci8200 and ci16200 instruments (Abbott Park, IL, USA). Reagents for the enzymatic creatinine method were from Abbott. Reagents for the immunoturbidimetric cystatin C method were from Gentian (Moss, Norway). Calculations of estimated glomerular filtration rate were performed with the CKD-epi formula according to Inker et al.[72]

4.2.3 Twin contact and age at separation

In study III & IV data on self-reported intra pair contact frequency, meaning the frequency by which the twins in a pair met each other, and age at separation was obtained from the SALT interviews. Data on contact frequency by at least one of the twins in a pair was available for 11 920 (97%) of the study participants. Contact frequency data was coded into 4 levels; (1) twins met each other less than once a year; (2) twins met on a yearly basis; (3) twins met on a monthly basis; (4) twins met on a weekly basis. Where both twins had reported age at separation, average value was used for analysis. By computing the rank-order correlation (Spearman) between contact frequency and the absolute intra-pair difference in adjusted trait-levels, we explored if contact frequency and the degree of shared-environment influences, such as age at separation from co-twin, was associated with similarity in trait levels.

4.2.4 DNA extraction and genotyping

DNA extraction for use in GCTA in study III was made using Puregene extraction kit (Gentra systems, Minneapolis, MN) on a 7 mL EDTA tube of blood. Subsequently DNA was stored

at _20°C. Subjects in whom the DNA concentration in the stock-solution was below 20 ng/L, as well as subset of 302 female monozygous twin pairs participating in a previous genome-wide effort was excluded. Thereafter, DNA from all available DZ twins+1 twin from each available MZ twin pair (n=9896) was sent to Uppsala, Sweden for genome-wide genotyping using the Illumina OmniExpress bead chip. Genotyping results for 9836 subjects and 731 442 autosomal SNPs passed the initial laboratory based quality control (QC). In further QC SNPs with missing information exceeding 3% (GENO>0.03) (n=3922), a minor allele frequency of less than 1% (n=79 893) or a Hardy-Weinberg equilibrium (HWE) test P value ≤1E-07 (n=3071), were excluded. Individuals with low genotyping success (MIND>0.03) (n=10), male heterozygosity of X-chromosomes (n=36), deviations in heterozygosity of more than 5 standard deviations from the population mean (n=49) and/or where unknown (cryptic) relatedness (n=124) was detected, were excluded. After the QC there were 9617 individuals and 644 556 autosomal SNPs remaining.

4.2.5 Estimating heritability

To estimate heritability for the chosen phenotypes in study III two different methods were used. First, a classic quantitative biometrical genetic model fitting method where the observed variation of each phenotype was decomposed into the influence of additive genetic factor (A), common environmental factor (C) or dominance genetic factor (D), and unique environmental factor (E).[136,139] The heritability was estimated as proportion of variance explained by A and D in an ADE model (if the intra-class correlation in MZ twins [rMZ] is larger than twice of the correlation in DZ twins [rDZ]), or only A in an ACE-model (if rMZ ≤ 2 * rDZ).

The second method used to estimate heritability was through genome-wide complex trait analysis (GCTA). Variance explained by all SNPs was estimated by restricted maximum likelihood (REML) modeling of the genetic relationship matrix (GRM) with phenotype-levels as implemented in the GCTA version 1.11 software package. [150] Since GCTA relies on comparisons between subjects that are not closely related, the sample was filtered for close relations. For complete monozygotic twin pairs, one twin was randomly selected to be genotyped. For complete dizygotic twin-pairs one member of each pair was randomly selected rendering the sample reduced to 6634 participants. A further restriction was implemented by only considering pair-wise combinations of unrelated subjects with relatedness less than 0.025, corresponding to relatedness between second and third cousins, which led to exclusion of 999, leaving n=5635 in the final sample on which GCTA-analysis was conducted. As there is a risk for bias arising from population stratification, i.e., variance

due to systematic ancestry differences due to migration, for example, [161] adjustment for genetic principal components (PCs) was performed.[162] Principal components of the genotype data significantly correlated to the phenotypes (cystatin C, creatinine and eGFR) were identified through a multiple stepwise regression analysis. As data on PCs for all individuals were not available (not all phenotyped subjects had been genotyped), a sub-analysis of all phenotypes adjusted for significant PCs were then made in order to investigate the magnitude of the influence from them.

4.2.6 Survival and hospitalization data

4.2.6.1 The Swedish National Patient Register

The Swedish National Board on Health and Welfare has provided data on in-patient care and hospital discharge diagnosis according to the International Classification of Disorders (ICD) codes in the National Patient Register since 1964. The National Patient Register includes hospitalized cases, as well as outpatient visits, but not visits to the primary care. External validation of the registry show high reliability with positive predictive values between 85-95% with low drop-out rates. The positive predictive value (i.e., validity) of the myocardial infarction diagnosis has been demonstrated to be 95% when only primary diagnoses are considered.[163] The validity of the stroke diagnosis in the registry has been reported to be 92%.[164]

4.2.6.2 The Cause of Death Register

All deceased Swedish citizens are registered in the Cause of Death Register. Data contains date, location, ICD-code and main contributory causes of death. The register is also maintained by the Swedish National Board on Health and Welfare. External validation has shown correct diagnosis in 77% in general, 87% for ischemic heart disease and 68% for cerebrovascular disease.[165]

4.2.7 Statistics

4.2.7.1 Study I

Levels of cystatin C, Creatinine clearance and GFR were compared between the study patients and the control group using t-test and correlation analysis. As the distribution of IL-6 and CRP levels were markedly skewed, non-parametric statistics were used to analyze these variables (Mann–WhitneyU-test). A backward stepwise multiple regression analysis was performed with cystatin C as dependent variable in the total population. The independent variables entered in the analysis were PAD, GFR, body mass index, age, waist circumference

HbA1c, 24 h pulse pressure (PP) and log IL-6. In order to analyze the separate effect of PAD on serum cystatin C-levels and the possible interaction between PAD and renal impairment an analysis of covariance (ANCOVA) was used. PAD and GFR-classification was entered as categorical independent variables and age, log IL-6 was entered as covariates in this analysis. Statistical analysis and database management were performed with Stat Soft, Inc. (2001). STATISTICA (data analysis software system), version 7. The authors had full access to the data and take responsibility for its integrity.

4.2.7.2 Study II

The PAD patients were divided into those with and without events and compared regarding biomarkers, ambulatory pulse pressure, and other variables. Further, the incidence of events was compared between high vs low tertiles of biomarkers and ambulatory pulse pressure. Because the distribution of the biomarkers NT-proBNP, hs-CRP, and cystatin C were skewed, logarithmic values were used in the final analyses. In addition, because a U-shaped relation between cystatin C levels and cardio vascular events was seen, a quadratic and centered term for cystatin C was analyzed. The predictive value of 24-hour pulse pressure , age, biomarkers, and relevant clinical variables were assessed by univariate Cox regression analysis, and hazard ratios for a 1 SD increase (with 95% CI) were calculated for 24-hour pulse pressure and logarithmic values of biomarkers. Ambulatory blood pressure variables, office blood pressure variables, log(hs-CRP), log(NT-proBNP), and log(cystatin C) were separately adjusted for basic cardiovascular risk factors (age, treatment with blood pressure-lowering drugs, and previous MI) in multivariable analysis. Further the biomarkers were separately adjusted for basic cardiovascular risk factors and 24-hour pulse pressure, day pulse pressure, night pulse pressure, and night systolic blood pressure, respectively, because these ambulatory blood pressure variables were significant predictors in a univariate analysis reported previously.[166] Finally, backward variable selection from all of the above (P to enter <0.05; P to remove >0.10) was performed to determine independent predictors in a multivariable Cox regression analysis. No interactions were found among included variables in interactional analysis. Statistical analysis and database management were performed with StatSoft (2010) STATISTICA data analysis software system version 9.1 (www.statsoft.com). Student t-tests, Mann–Whitney U tests, or $\chi 2$ tests were used for dependent or independent variables when appropriate. For linear correlation person r correlation coefficient was used.

4.2.7.3 Study III

Initial data handling and descriptive statistics were performed in SAS version 9.3 (SAS Institute, Cary, NC). To examine differences in variability and means between monozygotic

and dizygotic twins a t-test was performed. The distributions of cystatin C, creatinine and MDRD were skewed and these variables were log transformed in order to achieve approximate normal distributions. Before further investigations, traits for logarithmized cystatin C and creatinine together with machine estimated-GFR were adjusted for age and sex by linear regression models. In order to estimate GFR according to MDRD and the different CKD-epi formulas, age and sex were included in the calculations and thus no further adjustment was made for those covariates. After these adjustments, the residuals were z-score transformed and the influence of outliers was restrained through winsorizing outliers to -4 and +4 SDs.

In order to estimate variance components for each phenotype, maximum likelihood estimation and model fitting were performed using the structural equation statistical package OpenMx in R (http://openmx.psyc.virginia.edu). In univariate twin analyses the adjusted values of the investigated phenotypes were fitted into an ACE or ADE model.[167] We conducted a bivariate heritability analysis to estimate the relative importance of genetic, common, and unique environmental influence to the phenotypic correlation between cystatin C and creatinine. We also tested whether the genetic influence on cystatin C and creatinine were correlated to the genetic influence on cardiovascular morbidity in terms of manifest CVD. Based on the univariate models, an ACE model was preferred for CVD, whereas an ADE was preferred for cystatin C and creatinine. Because we cannot estimate the effect of A, D, C, E simultaneously with data from MZ and DZ twins only, ACE models were fitted for all bivariate twin analyses to keep consistency. Liability threshold model was applied to the dichotomous variable (CVD) by assuming that the ordered categories reflect an imprecise measurement of an underlying normal distribution of liability.[146] The variance of CVD was constrained to one for calculating its correlation with cystatin C/creatinine. Parameter estimates from a bivariate ADE model between cystatin C and creatinine can be accessed upon request. The genetic correlation (r_A) was calculated as: $cor_A/(\sqrt{(_A\%_{trait1})})*r_A*(\sqrt{(_A\%_{trait2})})$ where cor_A was standardized additive genetic covariance, $_A\%_{trait1}$ and $_A\%_{trait2}$ were the proportions of additive genetic variance for the respective traits. The common (r_C) and unique (r_E) environment component correlation was calculated similarly: $cor_C/(\sqrt{(_C\%_{trait1})})*r_C*(\sqrt{(_C\%_{trait2})})$ and $cor_E/(\sqrt{(_E\%_{trait1})})*r_E*(\sqrt{(_E\%_{trait2})})$. Through this the phenotypic correlation could be estimated to $cor_A+cor_C+cor_E$. Finally the bivariate heritability (h^2biv) was calculated as: $cor_A/(cor_A+cor_C+cor_E)$, which is the proportion of phenotypic correlation explained by genetic correlation.

4.2.7.4 Study IV

The predictive value per SD increase of logarithmized cystatin C for incident stroke, incident MI and incident ASCVD, was studied in a Cox-regression survival-analysis adjusted for sex, age, systolic blood pressure, diabetes, current smoking, eGFR (creatinine based CKD-epi), total cholesterol, HDL and anti-hypertensive medication. A robust sandwich covariance matrix estimate was incorporated into the model to account for any intra cluster dependence, which otherwise may inflate precision estimates due to correlated (twin-ships) data.

Same sexed twin pairs discordant for ASCVD and MI during follow up were identified. Independent two sample and paired t-tests were performed in order to verify significant differences regarding cystatin C levels on group- and pair level between twins with incident ASCVD and twins without incident ASCVD. Thereafter a conditional stepwise logistic regression analysis was performed in order to verify significant differences regarding cystatin C when adjusted for the same covariates as above. Sub-analyses were performed on twins discordant for coronary heart disease and stroke.

5 RESULTS

5.1 STUDY I

Baseline characteristics for the study populations are shown in table 1. Concentration of cystatin C was higher in PAD-patients compared to controls; 1.09±0.40 vs. 0.95±0.17 mg/L ($p < 0.001$). There was no significant difference in CCr or GFR between PAD-patients and control subjects. Both IL-6 and CRP concentration was higher in the PAD group.

Table 1
Main characteristics and laboratory investigations of the study population

	PAD-patients (*n* = 103)	Control subjects (*n* = 96)	p-value
Age (years)	68±8 (45–79)	68±8 (45–79)	
BMI (body mass index)	26.6±3.2 (20–35)	25.7±3.7 (18–35)	ns
Waist–hip ratio	0.96±0.05 (0.74–1.13)	0.94±0.05 (0.74–1.05)	$p < 0.01$
ABI (ankle brachial index)	0.66±0.19 (0.25–1.29)	1.11±0.11 (0.87–1.41)	*
History of hypertension	61	17	$p < 0.001$
Diabetes mellitus type II (yes)	16	7	ns
Clinical ischemic heart disease	44	0	*
Calculated creatinine clearance (mL/min)	81±27	82±22	$p = 0.78$
GFR (mL/min/1.73m2)	76±21	79±14	$p = 0.30$
Cystatin C (mg/L)	1.09±0.40	0.95±0.17	$p < 0.001$
CRP (mg/L)	2.63 (1.32, 4.87)	1.45 (0.72, 2.51)	$p < 0.001$
IL-6 (pg/mL)	1.60 (0.74, 2.85)	0.98 (0.37, 1.70)	$p < 0.01$
NT-proBNP (pg/mL)	167 (76, 418)	68 (38, 142)	$p < 0.001$
HbA1c (%)	4.8 (4.6, 5.2)	4.7 (4.5, 4.9)	$p < 0.001$
S-Cholesterol (mmol/L)	5.4±0.9	5.5±0.9	ns
HDL (mmol/L)	1.2±0.3	1.3±0.3	$p < 0.01$
LDL (mmol/L)	3.4±0.8	3.5±0.8	ns
S-Triglycerides (mmol/L)	1.7 (1.2, 2.2)	1.3 (0.9, 1.7)	$p < 0.01$
24-h SBP (mm Hg)	142±14	133±14	$p < 0.001$
24-h DBP (mm Hg)	78±8	79±8	ns

Values are expressed as mean±SD (range), median (interquartiles) or numbers.
* No statistics performed due to selection criteria.

When GFR was categorized into normal, mild, moderate and severe renal impairment, more PAD-patients had severe and moderate renal impairment as compared to control subjects ($p < 0.01$) (Table 2).

Table 2
Number of patients and controls categorized into normal, mild, moderate and severe renal impairment based on GFR

GFR (mL/min/1.73m2)	PAD (*n* = 103)	Controls (*n* = 96)	*p*-values
≥75 (normal)	61	55	< 0.01
60–74.9 (mild)	16	33	< 0.01
45–59.9 (moderate)	16	7	< 0.01
<45 (severe)	7	0	< 0.01

Table 3 shows the correlations for serum cystatin C-concentration with some clinical and laboratory variables in PAD-patients and control subjects. Cystatin C correlated to CCr in PAD-patients ($r = -0.60$, $p < 0.001$) as well as controls ($r = -0.44$, $p < 0.001$). There were positive correlations between cystatin C and log IL-6 in both groups (PAD $r = 0.35$, $p < 0.001$ and controls $r = 0.38$, $p < 0.001$). A positive correlation was also present between

cystatin C and log CRP in both groups (PAD, $r = 0.30$, $p < 0.01$ vs. $r = 0.32$, $p < 0.01$ among controls).

Table 3
Univariate correlation's between clinical variables and Cystatin concentration in PAD-patients and control subjects

	PAD-patients ($n = 103$)	Control subjects ($n = 96$)
Age	0.31 ($p < 0.01$)	0.34 ($p < 0.01$)
Body mass index	0.24 ($p < 0.05$)	0.04 (ns)
Waist circumference	0.24 ($p < 0.05$)	0.07 (ns)
24-h pulse pressure	0.24 ($p < 0.05$)	0.04 (ns)
Creatinine clearance (mL/min)	−0.61 ($p < 0.001$)	−0.44 ($p < 0.001$)
GFR (mL/min/1.73m2)	−0.78 ($p < 0.001$)	−0.58 ($p < 0.001$)
HbA1c	0.20 ($p < 0.05$)	−0.12 (ns)
HDL	−0.33 ($p < 0.01$)	−0.13 (ns)
log IL-6	0.35 ($p < 0.001$)	0.38 ($p < 0.001$)
log CRP	0.30 ($p < 0.01$)	0.32 ($p < 0.01$)
logNT pro BNP	0.57 ($p < 0.001$)	0.42 ($p < 0.001$)

Values are Pearson correlation coefficients (p-values)

In multivariate analysis using the combined population, PAD was a significant predictor of the cystatin C-concentration, as well as GFR. The results of this analysis are shown in Table 4.

Table 4
Backward stepwise multiple regression analysis of Cystatin C as dependent variable in the combined population ($n = 199$)

Dependent variable	Independent variables	Standard regression coefficient	Standard error of regression coefficient	Significance
Cystatin C	PAD	0.13	0.05	$p < 0.01$
$R = 0.76$, $p < 0.0001$	Waist circumference	0.10	0.05	$p < 0.05$
$F(4.182) = 62.9$	GFR	−0.67	0.05	$p < 0.001$
	log IL-6	0.14	0.05	$p < 0.001$

Values are expressed as number.

In an analysis of covariance where either GFR or calculated creatinine clearance, as well as log IL-6 were added as covariates, cystatin C-concentration remained higher in the PAD-group(Fig. 1). Further, analysis of covariance showed a significant interaction between PAD and GFR-classification on cystatin C-concentration (Fig. 2).

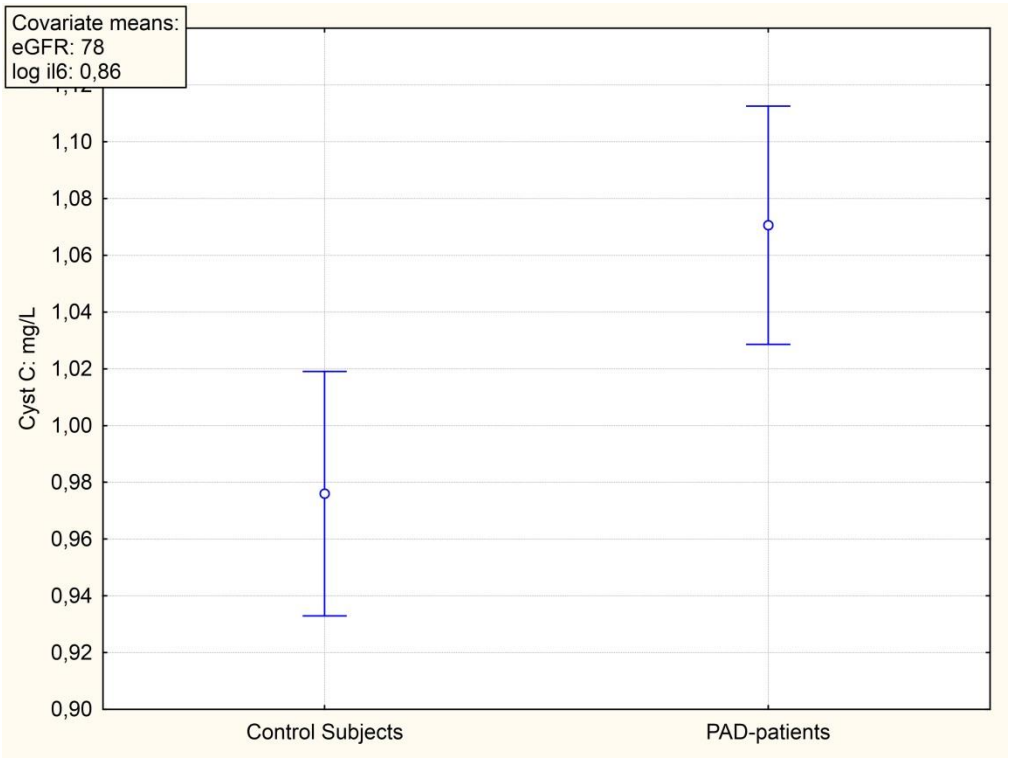

Figure 1. Cystatin C-concentration in PAD-patients (*n* = 103) and control subjects (*n* = 96). Analysis of covariance with covariates eGFR and log IL6. Current effect: $F(1.191) = 9.50$, $p = < 0.01$ (computed for covariates at their means). Vertical bars denote 0.95 confidence intervals (CI).

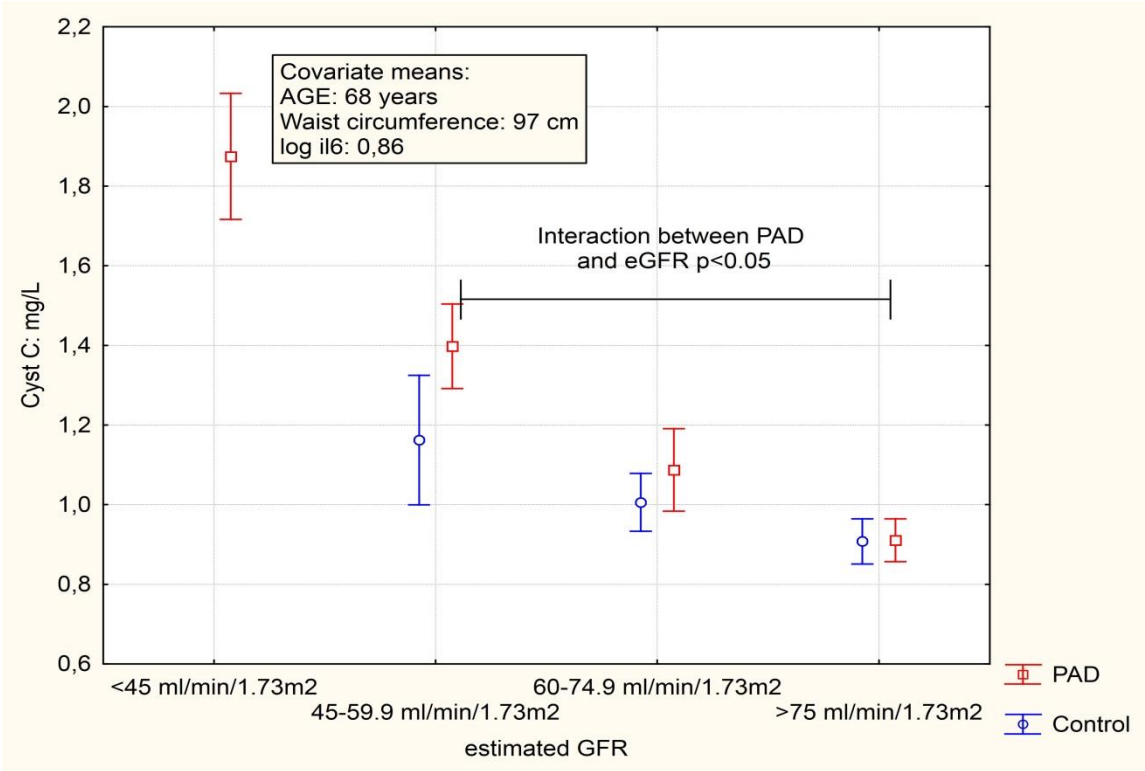

Figure 2. Interaction between PAD and eGFR-classification on Cystatin C-concentration. Analysis of covariance with covariates age, waist circumference and log IL6. Least Squares Means (computed for covariates at their means). Vertical bars denote 0.95 confidence intervals (CI).

5.2 STUDY II

Baseline patient characteristics are summarized in Table 5. The median observation time was 71 (range = 50–88) months. A total of 55 events occurred in 36 of 98 patients, including 14 AMI, 7 percutaneous coronary interventions (PCI), 9 coronary by-pass graft surgery (CABG), 10 strokes, and 15 cardiovascular (CV) deaths. A total of 8 events occurred in 7 of 90 control subjects (1 AMI, 2 PCI, 1 CABG, 3 strokes, and 1 CV death).

Table 5
Main characteristics and laboratory investigations of the study population (n = 188)

Characteristic	PAD patients without events (n = 62)	PAD patients with events (n = 36)	*p*-value	Control subjects (n = 90)
Age, y	67 ± 8	70 ± 6	0.02	68 ± 8
Smokers, current/former/never	14/45/3 (23/72/5)	9/24/3 (25/67/8)	0.73	15/40/35
ABI, ankle brachial index	0.67 ± 0.20	0.64 ± 0.18	0.41	1.11 ± 0.11
Duration of symptomatic IC	2 (1–7)	3.5 (1–10)	0.10	—
History of hypertension	33 (53)	26 (72)	0.06	14 (16)
Treatment w BP lowering drugs	38 (61)	32 (89)	0.004	19 (21)
ARB	6 (10)	1 (3)	0.20	2 (2)
ACE inhibitors	13 (21)	12 (33)	0.18	6 (7)
Beta-blockers	19 (31)	15 (42)	0.27	8 (9)
Calcium channel blockers	19 (31)	15 (42)	0.27	4 (4)
Diuretics	12 (19)	12 (33)	0.12	6 (7)
Diabetes mellitus type 2	8 (13)	8 (22)	0.23	7 (8)
Clinical ischemic heart disease	21 (34)	19 (53)	0.07	0
Previous AMI	10 (16)	13 (36)	0.03	0
Previous stroke	7 (11)	10 (28)	0.04	0
Estimated GFR, ml/min/1.73 m^2	77.4 ± 20.7	73.6 ± 22.7	0.41	80.2 ± 13.8
Cystatin C, mg/L	0.96 (0.96–1.06)	1.01 (0.86–1.33)	0.37	0.92 (0.82–1.01)
hs-CRP, mg/L	2.09 (1.04–4,00)	4.08 (1.87–7.11)	0.004	1.46 (0.74–2.56)
NT-proBNP, ng/L	119 (67–299)	409 (121–1,069)	<0.001	59 (36–123)
24-h SBP, mm Hg	140 ± 13	145 ± 17	0.13	133 ± 14
24-h DBP, mm Hg	79 ± 8	76 ± 8	0.08	79 ± 8
24-h pulse pressure, mm Hg	61 ± 10	68 ± 14	0.003	54 ± 10

Values are expressed as mean ± SD, median (interquartiles), or numbers (percentage). *P* values denote comparisons between peripheral artery disease (PAD) patients with events compared with PAD patients without events using Student *t* test, Mann–Whitney *U* test, or $\chi 2$ test where appropriate. Comparisons between PAD patients and control subjects have been reported previously. Abbreviations: 24-h, 24 hour; ACE, angiotensin-converting enzyme; AMI, acute myocardial infarction; ARB, angiotensin 2 receptor blocker; BP, blood pressure; DBP, diastolic blood pressure; GFR, glomerular filtration rate; IC, intermittent claudication; PAD, peripheral arterial disease; SBP, systolic blood pressure.

In PAD patients, higher values of NT-proBNP and hs-CRP were associated with higher incidence of CV events, whereas this was not the case for cystatin C. For NT-proBNP, the incidence of CV events was 64% vs. 15% ($P < 0.001$) in the high compared with the low tertile. For hs-CRP, the incidence was 55% vs. 21% ($P < 0.01$) and for cystatin C it was 51% vs. 42% ($P = 0.30$) (Figure 3).

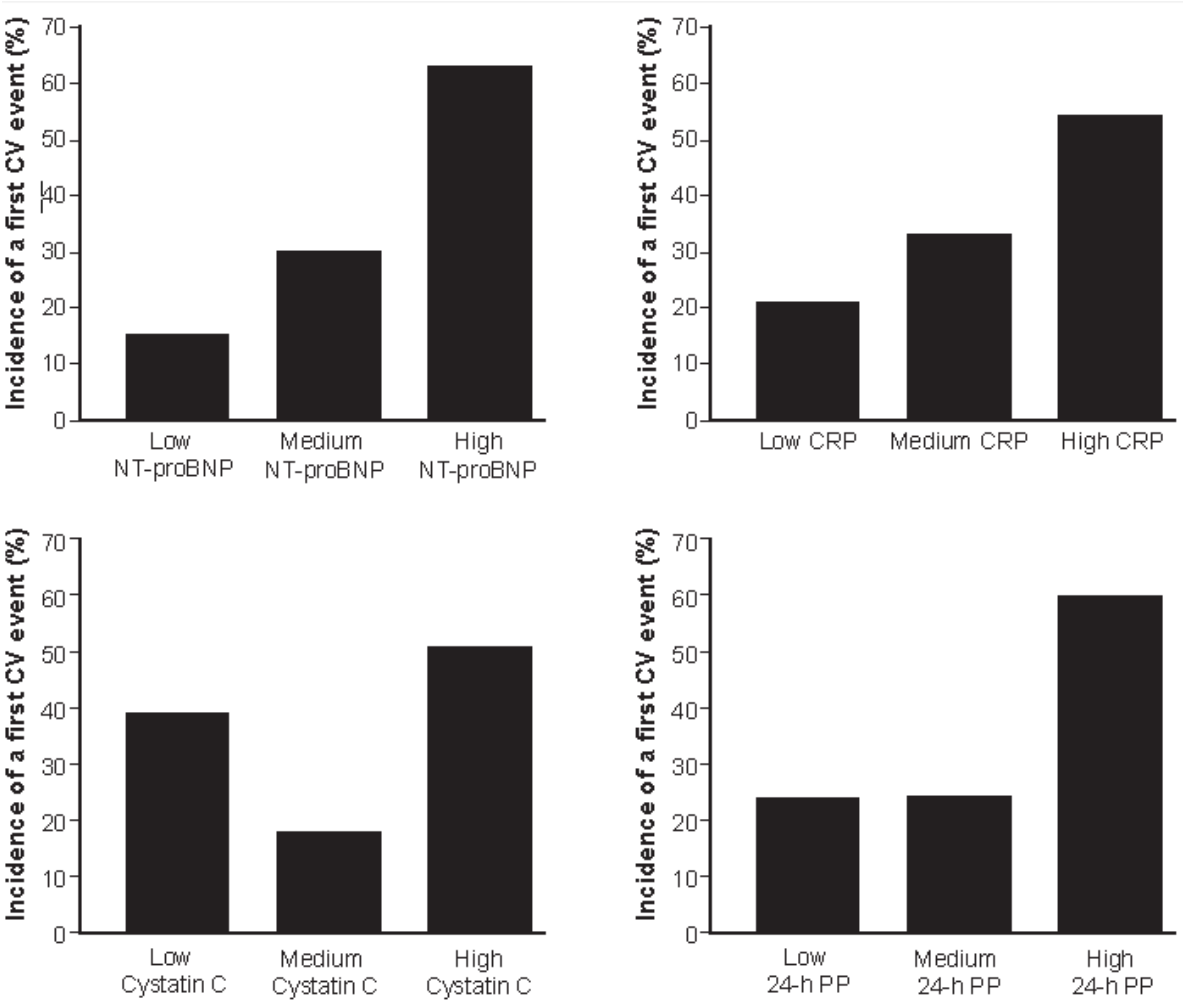

Figure 3. Incidence (%) of cardiovascular events in relation to tertiles of amino-terminal pro-B-type natriuretic peptide (NT-proBNP; low tertile: <97 ng/L; medium tertile: 97–319 ng/L; high tertile: >320 ng/L), high-sensitivity C-reactive protein (hs-CRP; low tertile: <1.7 mg/L; medium tertile: 1.7–4 mg/L; high tertile: >4 mg/L), cystatin C (low tertile: <0.89 mg/L; medium tertile: 0.89–1.06 mg/L; high tertile >1.06 mg/L), and 24-hour pulse pressure (24-h PP; low tertile: <58.7 mm Hg; medium tertile: 59–69 mm Hg; high tertile: >69 mm Hg). Comparison with $\chi 2$ for NT-proBNP high vs. low tertile: $P < 0.001$. Comparison for hs-CRP high vs. low tertile: $P < 0.01$. Comparison for cystatin C high vs. low tertile: $P = 0.30$. Comparison for 24-h PP high vs. low tertile: $P < 0.01$.

When used as continuous variables, 24-hour PP, log(NTproBNP),and log(hs-CRP) all predicted CV events in univariable analysis, whereas log(cystatin C) and centered quadratic term cystatin C did not. In multivariable analysis, log(NT-proBNP) and log(hs-CRP) still predicted CV events when adjusted for age, previous AMI, and treatment with BP-lowering drugs. Log(NT-proBNP) and log(hs-CRP) remained predictive for CV events when further adjusted for ambulatory blood pressure (Table 6).

Table 6
Hazard ratio (HRs) and 95% confidence intervals (CIs) for cardiovascular events in peripheral artery disease patients, uni- and multivariate (n = 98)

Predictor variables	HR	**HR 95%CI**	***P* value**
Univariate			
24-h pulse pressure, per SD	1,63	(1.18–2.24)	<0.01
Log(NT-proBNP), per SD	2.12	(1.49–3.00)	<0.001
Log(hsCRP), per SD	1.61	(1.20–2.16)	<0.01
Log(cystatin C), per SD	1.26	(0.91–1.74)	0.16
Centered quadratic term cystatin C	1.54	(0.96–2.48)	0.07
Multivariate adjusted for basic CV factors[a]			
24-h pulse pressure, per SD	1.44	(0.98–2.12)	0.06
Log(NT-proBNP), per SD	1.68	(1.09–2.60)	<0.05
Log(hsCRP), per SD	1.53	(1.13–2.08)	<0.01
Centered quadratic term cystatin C	1.27	(0.74–2.16)	0.39
Multivariate adjusted for basic CV factors and ABP[b].			
Log(NT-proBNP), per SD	1.62	(1.05–2.51)	<0.05
Log(hsCRP), per SD	1.63	(1.19–2.24)	<0.01
Centered quadratic term cystatin C	1.32	1.32	1.32

Abbreviations: 24-h, 24 hour; ABP, ambulatory blood pressure; AMI, acute myocardial infarction; BP, blood pressure; CV, cardiovascular;DBP, diastolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; NT-proBNP, amino-terminal pro-B-type natriuretic peptide; PAD,peripheral arterial disease; PP, pulse pressure; SBP, systolic blood pressure.
a Basic CV risk factors: age, treatment with BP-lowering drugs, previous AMI.
b Basic CV risk factors: age, treatment with BP-lowering drugs, previous AM and ABP.

5.3 STUDY III

Overall, 12,313 individuals were available for analysis, whereof 4,794 were in complete twin pairs. For the GCTA 5,635 individuals were included. General characteristics of the overall study group are summarized in Table 7. The mean age was 64.9 years, 55% of the sample was female, and 7.8% had a history of cardiovascular disease prior to enrollment.

Table 7
General Characteristics of Study Participants

	All	Men	Women
Total no of individuals[a](n)	12313	5585	6728
Complete pairs	4794	2133	2661
MZ[b](n)	3155	1353	1804
OSDZ[c](n)	4534	2191	2343
SSDZ[d](n)	4588	2018	2570
UKZ[e](n)	34	23	11
Age(years)	64.9(±8.1)	65.2(±8.0)	64.6(±8.2)
Weight (kg)	74.5(±13.9)	81.8(±12.3)	68.5(±12.1)
Height (cm)	169.2(±9.2)	176.3(±6.9)	163.2(±6.2)
Body Mass Index	26.0(±4.1)	26.3(±3.7)	25.7(±4.4)
Current Smoker(n)	2021(16.4%)	866(15.5%)	1155(17.2%)
Previous Smoker(n)	4870(39.6%)	2504(44.8%)	2366(35.2%)
Never Smoker(n)	5335(43.3%)	2170(38.9%)	3165(47%)
Diabetes	1202(9.8%)	687(12.3%)	515(7.7%)
Hypertension[f]	5901(47.9%)	2799(50.1%)	3102(46.1%)
Systolic Blood Pressure (mm Hg)	138.7(±19.7)	139.6(±19.3)	138(±20.0)
Diastolic Blood Pressure (mm Hg)	81.9(±11.0)	83.1(±10.6)	80.9(±11.3)
Pulse pressure (mm Hg)	56.8(±16.3)	56.5(±15.5)	57.1(±16.3)
Hyperlipidemia[g]	9094(73.9%)	3654(65.4%)	5440(80.9%)
Anti-hypertensive treatment	2675(21.7%)	1320(23.6%)	1355(20.1%)
Statin treatment	1648(13.4%)	911(16.3%)	737(11%)
CVD[g]	960(7.8%)	663(11.9%)	297(4.4%)
Waist circumference (cm)	91.3(±12.2)	97.0(±10.2)	86.6(±11.6)
Waist/Hip ratio	0.89(±0.13)	0.94(±0.13)	0.84(±0.11)

Values are in means ± SD or percentage [a]Number of individuals, [b]Monozygotic, [c]Opposite-sexed dizygotic, [d]Same-sexed dizygotic, [e]Unkonown Zygosity, [f]Systolic Blood Pressure >140 mmHg and/or Diastolic Blood Pressure >90 mmHg, [g]Cardiovascular Disease (Defined asICD10=I20.0,I21,I22,I63; ICD9=410,411B, 433,434; ICD8=410,411,432,433,434; Surgical codes=FNG02,FNG05,FNC,FND,FNE. diagnosed before study enrollment). [g]Total cholesterol >5.0 mmol/L

Mean cystatin C level was 1.02 mg/L and mean creatinine level was 77.5µmol/L. The estimates of GFR based on cystatin C were in general lower compared to estimates based on creatinine (Table 8).

Table 8
Clinical chemistry Characteristics of Study Participants

	All	Men	Women
N[a]	12313	5585	6728
Glucose (mmol/L)	5.6(±1.2)	5.8(±1.3)	5.4(±1.1)
Hba1c (%)	4.8(±0.7)	4.8(±0.7)	4.8(±0.6)
LDL (mmol/L)	3.8(±1.0)	3.7(±1.0)	3.9(±1.0)
HDL (mmol/L)	1.4(±0.4)	1.2(±0.3)	1.6(±0.4)
Triglycerides (mmol/L)	1.3(±0.8)	1.4(±0.9)	1.3(±0.7)
Total cholesterol (mmol/L)	5.8(±1.1)	5.5(±1.1)	6.0(±1.1)
CRP(mg/L)	3.22(±6.5)	3.37(±7.6)	3.08(±5.0)
Creatinine (µmol/L)	77.5(±23.6)	86.9(±28.8)	69.7(±14.2)
Cystatin C (mg/L)	1.02(±0.3)	1.05(±0.3)	0.99(±0.3)
Estimated GFR (MDRD)[a]	83.1(±18.1)	85.6(±19.8)	81.1(±16.2)
eGFR *CysC* (ml/min/1,73 m2)[b]	83.6 (±21.9)	81.2(±21.8)	85.7(±21.6)
CKD-epi *Crea* (ml/min/1,73 m2)[c]	86.1 (±16.0)	92.9 (±15.5)	80.4 (±14.0)
CKD-epi *CysC* (ml/min/1,73 m2)[c]	76.4 (±19.5)	77.2(±20.2)	75.8 (±18.8)
CKD-epi *Crea+CysC* (ml/min/1,73 m2)[c]	77.4 (±16.0)	76.8 (±16.2)	77.9 (±15.9)

All values are means ± Standard deviations. [a] GFR according to the MDRD formula. [b] Machine calculated GFR. [c] GFR according to the CKD-epi formula.

The estimated heritability (h^2) of cystatin C with the twin model was 0.55 (95% CI, 0.49-0.60) in men, 0.63 (95% CI, 0.59-0.66) in women and 0.60 (0.56-0.63) for both sexes combined. For creatinine h^2 was 0.56 (95%CI, 0.51-0.61) in men, 0.62 (95%CI, 0.58-0.65) in women and 0.59 (0.56-0.62) for both sexes combined. For these traits a dominant genetic component was significant. For the phenotypes derived from cystatin C and creatinine the dominance component was significant as well, whereas for CKD-epi estimated GFR and prevalent cardiovascular disease the correlation between MZ was less than twice the correlation for DZ and hence the ACE model was used. Effect of non-shared environment was significant for all phenotypic traits. The additive and dominant genetic variance components are presented in Table 9.

Mean contact level was 3.01 for MZ twins while it was 2.59 for DZ twins (t test, $p < .0001$) Mean age at separation 20.0 years and 18.6 years for MZ and DZ respectively (t-test, $p < .0001$). None of these measures were significantly related to the absolute intra-pair difference in adjusted trait levels (data not shown).

Table 9 Univariate ACE/ADE estimates for all phenotypes and for sex difference

Phenotype	Sex	h^2	A	C	D	E	Qualitative sex diff	Quantitative sex diff
Cystatin C	M	0.55 (0.49-0.60)	0.26 (0.14-0.43)		0.29 (0.11-0.43)	0.45 (0.40-0.51)	n	y
	F	0.63 (0.59-0.66)	0.56 (0.37-0.65)		0.06 (0.00-0.26)	0.37 (0.34-0.41)		
	Both	0.60 (0.56-0.63)	0.37 (0.24-0.49)		0.23 (0.09-0.37)	0.40 (0.37-0.44)		
Creatinine	M	0.56 (0.51-0.61)	0.24 (0.12-0.41)		0.32 (0.14-0.46)	0.44 (0.39-0.49)	n	y
	F	0.62 (0.58-0.65)	0.61 (0.49-0.65)		0.01 (0.00-0.13)	0.38 (0.35-0.42)		
	Both	0.59 (0.56-0.62)	0.38 (0.25-0.51)		0.21 (0.08-0.35)	0.41 (0.38-0.44)		
eGFR[a] *(Cys C)*	M	0.55 (0.50-0.60)	0.28 (0.14-0.47)		0.28 (0.65-0.42)	0.45 (0.40-0.50)	n	y
	F	0.63 (0.59-0.66)	0.60 (0.41-0.66)		0.03 (0.00-0.23)	0.37 (0.34-0.41)		
	Both	0.60 (0.57-0.63)	0.39 (0.26-0.52)		0.21 (0.07-0.35)	0.40 (0.37-0.43)		
MDRD[b]*(crea)*	M	0.62 (0.57-0.66)	0.39 (0.26-0.58)		0.22 (0.03-0.37)	0.38 (0.34-0.43)	n	y
	F	0.65 (0.62-0.68)	0.65 (0.58-0.68)		0.00 (0.00-0.07)	0.35 (0.32-0.38)		
	Both	0.63 (0.60-0.66)	0.56 (0.43-0.64)		0.07 (0.00-0.21)	0.37 (0.34-0.40)		
CKD-epi[c] *(Cys C+ crea)*	M	0.56 (0.46-0.62)	0.56 (0.46-0.62)	0.17 (0.11-0.25)		0.28 (0.25-0.31)	n	y
	F	0.36 (0.27-0.44)	0.36 (0.27-0.44)	0.39 (0.31-0.47)		0.25 (0.23-0.28)		
	Both	0.48 (0.42-0.54)	0.48 (0.42-0.54)	0.26 (0.20-0.31)		0.26 (0.24-0.28)		
CKD-epi[c] *(crea)*	M	0.62 (0.50-0.68)	0.62 (0.50-0.68)	0.06 (0.02-0.16)		0.32 (0.28-0.36)	n	y
	F	0.37 (0.27-0.48)	0.37 (0.27-0.48)	0.30 (0.21-0.39)		0.32 (0.29-0.36)		
	Both	0.54 (0.47-0.61)	0.54 (0.47-0.61)	0.14 (0.08-0.19)		0.32 (0.30-0.35)		
CKD-epi[c] *(Cys C)*	M	0.51 (0.39-0.58)	0.51 (0.39-0.58)	0.21 (0.15-0.31)		0.28 (0.25-0.32)	n	y
	F	0.38 (0.30-0.47)	0.38 (0.30-0.47)	0.38 (0.30-0.46)		0.24 (0.21-0.26)		
	Both	0.46 (0.40-0.52)	0.46 (0.40-0.52)	0.28 (0.23-0.33)		0.25 (0.24-0.28)		
CVD[d]	M	0.39 (0.02-0.67)	0.39 (0.02-0.67)	0.17 (0.05-0.45)		0.44 (0.32-0.58)	n	n
	F	0.20 (0.00-0.61)	0.20 (0.00-0.61)	0.27 (0.00-0.51)		0.53 (0.37-0.70)		
	Both	0.41 (0.13-0.62)	0.41 (0.13-0.62)	0.12 (0.00-0.31)		0.47 (0.38-0.59)		
CAE[e]	M	0.48 (0.08-0.77)	0.48 (0.08-0.77)	0.19 (0.00-0.49)		0.33 (0.22-0.48)	n	n
	F	0.30 (0.00-0.72)	0.30 (0.00-0.72)	0.25 (0.00-0.58)		0.45 (0.27-0.66)		
	Both	0.51 (0.21-0.73)	0.51 (0.21-0.73)	0.12 (0.00-0.33)		0.37 (0.27-0.49)		
STROKE[f]	M	0.24 (0.00-0.61)	0.24 (0.00-0.61)	0.17 (0.00-0.47)		0.60 (0.38-0.80)	n	n
	F	0.48 (0.02-0.70)	0.48 (0.02-0.70)	0.00 (0.00-0.33)		0.52 (0.30-0.78)		
	Both	0.45 (0.07-0.59)	0.45 (0.07-0.59)	0.00 (0.00-0.00)		0.55 (0.41-0.72)		

h^2=heritability, defined as A in ACE-model and A+D in ADE-model; A=added genetic component, C=common environmental component, D=dominance genetic component, E=unique environment component; Qualitative sex-difference= genetic correlation of less than .5 among opposite-sex twin pairs suggesting different genetic factors operating for males and females; Quantitative sex-difference=significant difference in correlation between male and females. [a] Machine calculated GFR. [b] GFR according to the MDRD formula. [c] GFR according to the CKD-epi formula.[d]Cardiodascular disease (definition in Method). [e]Coronary artery event (definition in method.) [f]Stroke defined in method.

Through GCTA we found the estimate of the proportion of genetic variance to total phenotypic variance, V(g)/V(p), captured by all investigated markers to be significant for all traits (Table 10). GCTA heritability for cystatin C was 0.40 (SE 0.07, p $8e^{-9}$) and for creatinine 0.19 (SE 0.07, p 0.003). As there were sex-differences in heritability observed in the classical twin-model, we tested a GCTA model that included gene by sex interaction without finding any significant interaction term (p>0.05) for any of the 7 tested phenotypes.

Table 10
GCTA analysis

Phenotype	Source	Variance	SE	P-value
Cystatin C	V genotypic (g)	0.404	0.0763	
	V environmental (e)	0.608	0.0749	
	V phenotypic (p)	1.012	0.0192	
	V (g) / V (p)	**0.399**	**0.0743**	**$8e^{-9}$**
Creatinine	V genotypic (g)	0.190	0.0727	
	V environmental (e)	0.798	0.0733	
	V phenotypic (p)	0.988	0.0186	
	V (g) / V (p)	**0.192**	**0.0733**	**0.003**
eGFR(Cys C)[a]	V genotypic (g)	0.418	0.0766	
	V environmental (e)	0.594	0.0751	
	V phenotypic (p)	1.012	0.0192	
	V (g) / V (p)	**0.413**	**0.0644**	**$3e^{-9}$**
MDRD(Crea)[b]	V genotypic (g)	0.186	0.0720	
	V environmental (e)	0.791	0.0726	
	V phenotypic (p)	0.977	0.0184	
	V (g) / V (p)	**0.191**	**0.0734**	**0.004**
Cdk-epi(Cys C+Crea)[c]	V genotypic (g)	0.192	0.0737	
	V environmental (e)	0.821	0.0744	
	V phenotypic (p)	1.013	0.0191	
	V (g) / V (p)	**0.190**	**0.0725**	**0.003**
Cdk-epi(Cys C)[c]	V genotypic (g)	0.247	0.0733	
	V environmental (e)	0.769	0.0735	
	V phenotypic (p)	1.016	0.0192	
	V (g) / V (p)	**0.243**	**0.0717**	**$9e^{-5}$**
Cdk-epi(Crea)[c]	V genotypic (g)	0.084	0.0707	
	V environmental (e)	0.918	0.0724	
	V phenotypic (p)	1.002	0.0189	
	V (g) / V (p)	**0.084**	**0.0705**	**0.1**

All values adjusted for age, sex and correlated principal components.SE= Standard error, V=variance. [a] GFR according to the MDRD formula. [b] Machine calculated GFR. [c] GFR according to the CKD-epi formula.

The results of the bivariate heritability analysis are shown in Table 11 . The phenotypic correlation between cystatin C and creatinine was estimated to 0.63 (95%CI, 0.61-0.65) in men and 0.55 (95%CI, 0.53-0.57) in women. The proportion of this correlation explained by additive genetic components (the bivariate heritability, (h2biv)) was 0.52 (95%CI, 0.44-0.59) in men and 0.51 (95% CI, 0.36-0.65) in women. For cystatin C vs CVD the correlation was

0.16 (95% CI, 0.12-0.20) in men and 0.17 (95%CI, 0.13-0.21) in women and the genetic correlation in males was 0.41 (0.21-0.62) while it was non-significant in females.

Table 11 Bivariate Heritability analysis

	Male	Female
Bivariate Correlations	**Cystatin C vs Creatinine**	
Genetic (r_a)	0.64 (0.58,0.70)	0.53 (0.43,0.62)
Shared environmental (r_c)	-0.99 (-1,1)	0.99 (-1,1)
Non-shared environmental (r_e)	0.63 (0.58-0.67)	0.52 (0.47-0.57)
Phenotypic correlation	0.63 (0.61,0.65)	0.55 (0.53,0.57)
mediated by:		
Bivariate heritability (biv h^2)	0.52 (0.44,0.59)	0.51 (0.36,0.65)
Bivariate shared environment (biv c^2)	0.00 (-0.02,0.04)	0.12 (-0.00,0.25)
Bivariate non-shared environment (biv e^2)	0.48 (0.41,0.56)	0.37 (0.32,0.42)
Bivariate Correlations	**Cystatin C vs CVD**	
Genetic (r_a)	0.41 (0.21-0.62)	0.05 (-0.24, 0.43)
Shared environmental (r_c)	-0.99 (-1,1)	0.99 (-1,1)
Non-shared environmental (r_e)	0.01 (-0.12, 0.13)	0.17 (0.06-0.28)
Phenotypic correlation	0.16 (0.12-0.20)	0.17 (0.13-0.21)
mediated by:		
Bivariate heritability (biv h^2)	1.13 (0.59,1.72)	0.10 (-0.49,0.88)
Bivariate shared environment (biv c^2)	-0.15 (-0.49,0.17)	0.43 (-0.20,0.89)
Bivariate non-shared environment (biv e^2)	0.02 (-0.35, 0.39)	0.47 (0.17,0.77)
Bivariate Correlations	**Creatinine vs CVD**	
Genetic (r_a)	0.32 (0.12-0.54)	-0.19 (-0.36,0.06)
Shared environmental (r_c)	-0.99 (-1,1)	0.43 (-0.38, 1)
Non-shared environmental (r_e)	-0.11 (-0.23, 0.02)	0.09 (-0.33, 0.21)
Phenotypic correlation	0.09 (0.05,0.13)	0.05 (0.01,0.09)
mediated by:		
Bivariate heritability (biv h^2)	1.64 (0.64,3.03)	-1.16*
Bivariate shared environment (biv c^2)	-0.09 (-0.79,0.55)	1.37*
Bivariate non-shared environment (biv e^2)	-0.55 (-1.50,0.08)	0.79*

Cystatin C and creatinine are sex-, age adjusted and log-transformed.*****There were some convergence problems when estimating the confidence interval for creatinine vs. CVD in females.

5.4 STUDY IV

11402 participants were followed for a median time of 71 (SD±16) months, (general characteristics are shown in Table 12). 119 monozygotic and 155 same sexed dizygotic twin pairs, with no prevalent ASCVD at study enrollment, became discordant for incident ASCVD, stroke or MI during follow-up.

Table 12
General Characteristics of Study Participants

	All	Women	Men
Total number of individuals* (n)	11402	6455(57%)	4947(43%)
Complete Pairs	4127	4781(58%)	3473(42%)
Monozygotic Twins (n)	2879	1680(58%)	1199(42%)
Same Sex Dizygotic Twins(n)	4299	2511(58%)	1788(42%)
Opposite Sex Dizygotic Twins(n)	4194	2255(54%)	1939(46%)
Unkonown zygosity (n)	30	9(30%)	21(70%)
Age (years)	64.5(±8.0)	64.3(±8.1)	64.7(±7.9)
Height(cm)	169.1(±10.7)	163.2(±8.0)	176.4(±9.0)
weight (Kg)	74.2(±13.8)	68.5(±12.1)	81.7(±12.2)
Body Mass Index (kg/m2)	25.9(±4.1)	25.7(±4.4)	26.2(±3.7)
Systolic Blood Pressure (mmHg)	138.6(±19.7)	137.9(±20.0)	139.5(±19.2)
Glucose mmol/L (serum)	5.5(±1.1)	5.4(±1.0)	5.7(±1.3)
HbA1c % (serum)	4.8(±0.6)	4.8(±0.6)	4.8(±0.7)
HDL Cholesterol mmol/L (serum)	1.4(±0.4)	1.6(±0.4)	1.3(±0.3)
LDL Cholesterol mmol/L (serum)	3.8(±1.0)	3.9(±1.0)	3.8(±0.9)
Total Cholesterol mmol/L (serum)	5.9(±1.1)	6.0(±1.1)	5.6(±1.1)
Cystatin C mg/L (Plasma)	1.00(±0.25)	0.99(±0.23)	1.03(±0.27)
Creatinine µmol/L (Plasma)	76.5(±18.1)	69.5(±12.6)	85.8(±20.0)
eGFR mL/min/1,73 m2 (CKD-epi)[†]	86.3(±15.6)	80.7(±13.8)	93.6(±14.8)
Current Smoker(n)	1861(16%)	1098(17%)	763(15%)
Anti-hypertensive tretment (n)	2459(21.5%)	1426(22%)	1033(21%)
Anti-lipids treatment (n)	1046(9%)	582(9%)	464(9%)
Diabetes[‡] (n)	933(8%)	421(6.5%)	512(10%)

Values are in means ± SD or percentage *Number of individuals, [†]Derived from the CDK-epi formula based on creatinine
[‡]According to Swedish diabetes registry

The results of Cox regression analysis in the whole cohort are shown in Table 13. In univariate analysis cystatin C was a predictor of incident stroke ((HR, 95% CI) 1.69, 1.56-1.84) MI (1.49, 1.39-1.60) and ASCVD (1.57, 1.47-1.67). No association between MI and creatinine based CKD-epi was observed. When adjusted for all covariates including CKD-epi calculated eGFR(model 2, Table 13), cystatin C remained a predictor of incident stroke (1.45, 1.25-1.70), MI (HR 1.16, CI 1.01-1.33) and ASCVD (1.26, 1.13-1.41)

Table 13
Hazard ratios for incident ASCVD in unadjusted and adjusted cox prediction models in 11 402 twins

	Univariate		Adjusted Model 1*		Adjusted Model 2†	
Variable	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Log Cystatin C						
Stroke	1.69 (1.56-1.84)	<.001	1.31(1.17-1.46)	<.001	1.45 (1.25-1.70)	<.001
MI	1.49 (1.39-1.60)	<.001	1.11 (1.00-1.24)	.05	1.16 (1.01-1.33)	.04
ASCVD	1.57 (1.47-1.67)	<.001	1.19(1.10-1.29)	<.001	1.26 (1.13-1.41)	<.001
CDK-epi (crea)						
Stroke	0.78(0.69-0.87)	<.001	0.90 (0.80-1.03)	.13		
MI	0.94 (0.85-1.04)	.25	0.96 (0.86-1.08)	.54		
ASCVD	0.99 (0.99-1.00)	<.001	0.94 (0.86-1.03)	.17		
Age						
Stroke	1.11 (1.09-1.12)	<.001				
MI	1.07 (1.06-1.08)	<.001				
ASCVD	1.09 (1.08-1.10)	<.001				
Sex						
Stroke	0.51 (0.41-0.63)	<.001				
MI	0.38 (0.31-0.46)	<.001				
ASCVD	0.44 (0.37-0.51)	<.001				
Smoke						
Stroke	1.03 (.96-1.12)	.39				
MI	1.10 (1.03-1.18)	.003				
ASCVD	1.07 (1.10-1.31)	.006				
HDL						
Stroke	0.67 (0.51-0.89)	.006				
MI	0.33 (0.26-0.43)	<.001				
ASCVD	0.48 (0.39-0.59)	<.001				
Total Cholesterol						
Stroke	0.91 (0.82-1.02)	0.09				
MI	1.01 (0.92-1.12)	0.81				
ASCVD	0.98 (0.91-1.06)	0.60				
Anti-HT treat						
Stroke	0.91 (0.82-1.02)	0.09				
MI	1.01 (0.92-1.12)	0.81				
ASCVD	0.98 (0.91-1.06)	0.60				
Diabetes						
Stroke	0.91 (0.82-1.02)	0.09				
MI	1.01 (0.92-1.12)	0.81				
ASCVD	0.98 (0.91-1.06)	0.60				

*Adjusted model 1 includes age, sex, SBP, serum cholesterol, HDL, treatment for hypertension (yes/no), diabetes (yes/no),) and smoking status (yes/no),
†Adjusted model 2 includes age, sex, SBP, serum cholesterol, HDL, treatment for hypertension (yes/no), diabetes (yes/no), smoking status (yes/no) and eGFR(CKD-epi)

A total of 116 monozygotic (MZ) and 155 same sexed dizygotic (DZ) twin pairs became discordant for incident ASCVD during follow-up. In twins discordant for stroke, cystatin C was higher in diseased compared to healthy twins in both MZ (1.11 +/- 0.27 mg/L vs 1.06 +/- 0.26 mg/L, $p<0.05$) and DZ pairs (1.2 +/- 0.37 mg/L vs 1.07 +/- 0.23 mg/L, $p<0.01$) whereas no difference was observed for creatinine (Table 14). DZ twins also showed significant intra-pair difference in cystatin C levels with regard to discordance in the ASCVD endpoint (1.13 +/- 0.31 mg/L vs 1.06 +/- 0.22 mg/L, $p<0.02$).

Table 14
Paired T-tests in twin-pairs discordant for stroke, ASCVD and MI

Stroke

	MZ (n=59)			DZ (n=79)		
Variable	Sick	Healthy	p-value	Sick	healthy	p-value
Cystatin C	1.11+/-0.27	1.06+/-0.26	***0.0439***	1.20+/-0.37	1.07+/-0.23	***0.0015***
Creatinine	76.12+/-16.87	79.39+/-20.32	0.1288	85.05+/-20.19	81.16+/-13.42	0.1411
CKD-EPI	85.56+/-17.32	83.51+/-16.91	0.1902	82.52+/-16.32	79.73+/-18.15	0.1235
HDL	1.40+/-0.48	1.34+/-0.35	0.1431	1.36+/-0.38	1.43+/-0.42	0.1377
Total Cholesterol	5.98+/-1.30	5.77+/-1.26	0.2675	5.90+/-1.29	5.91+/-1.37	0.9454
BMI * †	26.66+/-3.93	26.09+/-4.49	0.1487	25.84+/-3.86	25.58+/-3.75	0.5813
SBP ‡ §	149.2+/-21.92	146.1+/-20.22	0.5433	151.6+/-25.79	147.0+/-21.32	0.1280

*MZ(n=54) †DZ(n=75) ‡MZ(n=52) §DZ(n=74)

MI

	MZ (n=71)			DZ (n=84)		
Variable	Sick	Healthy	p-value	Sick	healthy	p-value
Cystatin C	1.06+/-0.22	1.06+/-0.22	0.9359	1.11+/-0.32	1.08+/-0.24	0.5786
Creatinine	77.28+/-16.86	79.97+/-18.32	0.1273	81.66+/-16.32	80.57+/-16.98	0.5189
CKD-EPI	86.02+/-16.03	83.42+/-15.88	0.1309	84.38+/-16.52	83.55+/-16.69	0.6463
HDL	1.29+/-0.35	1.30+/-0.38	0.7270	1.30+/-0.38	1.38+/-0.38	0.0973
Total Cholesterol	5.78+/-1.06	5.62+/-1.06	0.3142	6.11+/-1.22	5.92+/-0.99	0.2587
BMI * †	26.13+/-3.83	26.56+/-3.84	0.1818	26.51+/-3.86	25.82+/-4.05	0.1858
SBP * ^	143.3+/-20.27	149.0+/-19.68	**0.0481**	147.6+/-23.98	146.2+/-20.63	0.7143

*MZ(n=61) †DZ(n=77) ‡MZ(n=67) §DZ(n=80)

CVD

	MZ (n=116)			**DZ (n=149)**		
Variable	Sick	Healthy	p-value	Sick	healthy	p-value
Cystatin C	1.08+/-0.25	1.07+/-0.24	0.3332	1.13+/-0.31	1.06+/-0.22	***0.0182***
Creatinine	76.71+/-16.72	80.11+/-19.43	***0.0210***	81.66+/-18.46	80.68+/-15.58	0.7129
CKD-EPI	85.86+/-16.63	83.19+/-16.37	***0.0310***	82.70+/-16.78	84.31+/-19.45	0.5561
HDL	1.33+/-0.36	1.31+/-0.30	0.3263	1.33+/-0.34	1.40+/-0.39	0.0502
Total Cholesterol	5.92+/-1.16	5.69+/-1.14	0.0692	5.98+/-1.25	5.88+/-1.18	0.4428
BMI * †	26.45+/-4.21	26.46+/-3.93	0.8910	26.08+/-3.58	25.69+/-3.82	0.3010
SBP * †	146.6+/-21.19	148.5+/-19.74	0.4118	147.6+/-23.98	146.2+/-20.63	0.3276

*MZ(n=107) †DZ(n=139)

The results of conditional regression analysis in pairs discordant for incident ASCVD are shown in Table 15. When adjusted for the same covariates as in the cox regression model and stratified by zygosity cystatin C did not remain significantly associated with any outcome. In univariate analysis cystatin C was significantly associated with stroke and ASCVD but not MI in same sexed dizygotic twins. Univariate analysis of cystatin C in MZ did not reach significance level (p=0.052). However in multivariate analysis of cystatin C and eGFR cystatin C was significantly associated with incident stroke in MZ, although the confidence interval was quite broad.

Table 15
Odds ratios per 1SD increase for ASCVD in discordant MZ and same sex DZ twin pairs

	MZ						DZ					
	Univariate		Adjusted model 1*		Adjusted Model 2†		Univariate		Adjusted model 1*		Adjusted Model 2†	
Variable	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Log Cystatin C												
Stroke	2.33 (0.99-5.47)	0.052	2.33 (0.72-7.58)	0.16	4.93 (1.01-24.09)	**0.049**	2.06(1.25-3.39)	**0.005**	1.88(1.11-3.19)	**0.019**	1.86(1.01-3.43)	**0.046**
MI	0.91 (0.09-9.51)	0.935	0.34 (0.01-16.19)	0.58	2.03(0.01-352.6)	0.79	1.61(0.30-8.60)	0.755	0.81(0.07-9.14)	0.87	0.78 (0.06-9.72)	0.84
ASCVD	1.18 (0.76-1.83)	0.469	1.03(0.39-2.69)	0.96	1.24(0.37-4.16)	0.73	1.39(1.05-1.85)	**0.021**	1.45(0.91-2.30)	0.12	1.41(0.85-2.35)	0.19

*Adjusted model 1 includes SBP, serum cholesterol, HDL, diabetes (yes/no), anti hypertensive treatment (yes/no) and smoking status (yes/no),

†Adjusted model 2 includes SBP, serum cholesterol, HDL, , treatment for hypertension (yes/no), diabetes (yes/no), smoking status (yes/no), and eGFR(CKD-epi)
Age and Sex inherent in all models.

Data on contact frequency by at least one of the twins in a pair was available for 11040 (97%) of the study participants. The intra pair correlation on contact frequency was high (rho=0.80) for the 3954 pairs where both responded. Data on age at separation was available for 11145 (98%) individuals, correlation was somewhat lower compared to contact frequency, (rho=0.65) for 3206 responding pairs. MZ twins reported a higher contact frequency and higher mean age at separation than DZ twins. Mean contact level was 3.02 (SD+/-0.86) for MZ twins while it was 2.72 (SD+/-0.84) for DZ twins (t test, $p < .0001$). Mean age at separation 19.49 (SD+/-3.38) years and 18.45 (SD+/-3.48) years for MZ and DZ respectively (t-test, $p < .0001$). None of these measures were significantly related to the absolute intra-pair difference in adjusted trait levels in MZ, but contact level was related to intra-pair difference in cystatin C levels in DZ (Table 16).

Table 16
Correlation Between Absolute Intra-Pair Difference of Adjusted Trait Values and (A) Co-Twin Contact Frequency and (B) Age at Separation From Co-Twin

Phenotype	MZ[†]			ssDZ[‡]		
	r[*]	p-value	N(pairs)	r[*]	p-value	N(pairs)
(A) Contact frequency						
Cystatin C	-.04	.16	1173	**-.06**	**.03**	1527
CKD-epi	-.01	.65	1165	-.03	.22	1530
(B) Age at Separation						
Cystatin C	-.01	.70	1177	-.03	.25	1495
CKD-epi	-.01	.65	1116	-.003	.90	1499

[*]Spearman correlation coefficient, Note: p-values remained unsignificant for opposite sex dizygotic when stratified by sex
[†]Monozygotic twins
[‡]Same Sex Dizygotic twins

6 DISCUSSION

The main purpose of this thesis was to study the association between cystatin C and atherosclerotic cardiovascular disease with a special focus on the relative importance of genetics and environment. In paper I, we observed that the mean serum level of cystatin C- was higher in PAD patients compared with healthy controls even when corrected for differences in eGFR, IL-6 and CRP. In a follow-up, cystatin C was not a significant predictor of incident cardiovascular events in patients with manifest PAD, although we observed a U-shaped relation between tertiles of cystatin C-concentration and outcome.

In a large twin study we observed a higher heritability of cystatin C compared with previous studies on the matter. The GCTA analysis provided independent evidence for significant heritability indicated by the twin-model. We also observed a significant genetic correlation between levels of cystatin C and creatinine as well as a genetic correlation between levels of cystatin C and CVD in males indicating an overlap of the genetic factors that influence the two traits in males. In a prospective study in the same population we showed that cystatin C was a predictor for incident ASCVD, confirming previous studies. A novel finding was that cystatin C remained a predictor for incident stroke after adjustment for genetic confounding, in identical twins discordant for incident stroke, highlighting the importance of unique environmental factors for the association between cystatin C and ASCVD.

6.1 CYSTATIN C AND THE ASSOCIATION TO PAD

In our first study we observed a higher mean level of cystatin C in PAD patients compared with a matched control group. The major underlying cause of PAD, atherosclerosis, is an inflammatory disease of the cardiovascular system, which is characterized by extensive remodeling of the extracellular matrix of the arterial wall. During this process several pathological events take place, such as proteolysis, translocation of leukocytes through the basement membrane, migration of smooth muscle cells through the elastic laminas, and finally, disruption of the vessel wall into aneurysms or occlusive plaques.[168] These processes create a local inflammatory response with the activation of growth factors and cytokines, of which IL-6 is of major importance as a mediator of the inflammatory process.[169] It has been implicated that an imbalance between the expression of cathepsines and their endogenous inhibitor cystatin C is another important mechanism in atherogenesis.[170] In contrast to the findings for cystatin C we observed no significant difference in eGFR estimated by MDRD or calculated creatinine clearance between patients

and controls. This is in agreement with the fact that creatinine is a rather insensitive GFR marker.[64,67,171] In an analysis of covariance where either eGFR or calculated creatinine clearance was added as a covariate, cystatin C concentration remained higher in the PAD-group. Thus, our results indicated that the increase in cystatin C concentration seen in PAD-patients might not be related solely to a concomitant decline of kidney function. One hypothesis is that the increased cystatin C concentration may be related to a more advanced stage of atherosclerosis and therefore we also adjusted for inflammatory markers related to atherosclerosis. We found that the increased level of cystatin C in PAD patients persisted when either IL-6 or CRP levels were added as covariates in a multivariate model, lending further support to the hypothesis that cystatin C could be used as an independent marker of atherosclerotic disease. Cystatin C-concentration correlated to IL-6 and CRP concentrations in PAD-patients but variation in IL-6 levels or CRP could not explain all of the increased concentration of cystatin C seen in PAD-patients. Thus, it seems that the observed increase in cystatin C-concentration could reflect other mechanisms in the atherosclerotic process than markers of inflammation such as CRP and IL-6 do. Such possible mechanisms may include, atherosclerotic vascular wall remodeling by collagenolysis and elstinolysis or a more rapid progression of vascular ageing.

6.2 PREDICTIVE VALUE OF CYSTATIN C IN PAD

In paper II we studied the predictive value of cystatin C in PAD-patients. The main findings was that cystatin C was not a predictor of a combined endpoint consisting of mortality or any hospitalization due to acute myocardial infarction , stroke or coronary revascularization percutaneous coronary intervention or coronary artery by-pass graft. Our findings are in contrast to the results from a longitudinal study in a somewhat bigger cohort, but with comparable baseline characteristics as ours, which suggested that cystatin C was a predictor of 5-year cardiovascular mortality independent of renal function in PAD patients.[171] Although the different findings in the two studies are surprising one possible explanation may be that different outcomes were studied. Besides the study by Urbonaviciene et al, there are very few studies regarding the prospective relationship between cystatin C and CVD in patients with PAD. Both Koenig et al and Ix et al have previously reported a predictive value of cystatin C in populations with general atherosclerotic cardiovascular disease.[6,172] Admittedly, the last mentioned prospective studies were partly different from ours both in terms of study size and study population. The Ix-cohort consisted of individuals with stable coronary heart disease defined as history of MI, angiographic coronary stenosis, stress-induced myocardial ischemia or a history of coronary revascularization, while the Koenig-

cohort consisted of persons with prevalent coronary heart disease. Regarding the endpoints, the Ix-endpoint was all-cause mortality, cardiovascular events (AMI and stroke) and incident heart failure, while Koenig's was more similar to ours with CVD death or non-fatal MI or ischemic cerebrovascular event. Nevertheless, peripheral arterial disease is a manifestation of general atherosclerotic disease that is often silent and thus underdiagnosed even in patients with manifest ischemic heart disease.[173,174] Hence it is not farfetched to draw the conclusion that a considerable comorbidity might be present and thus the patients in the Ix and Koenig studies might very well have suffered from concomitant asymptomatic undiagnosed peripheral atherosclerosis.

A plausible explanation to the result in our study is the small size of the cohort and the rather few events which might give rise to a type 2 error, however for other biomarkers as well as ambulatory blood pressure, we could report highly significant associations to the outcome . Lastly, the finding of an U-shaped relation between tertiles of cystatin C concentration and outcome (Figure 3) was not anticipated, because of this a quadratic model was also used without any change of significance of the result. The U-shaped relation between cystatin C concentration and a second cardiovascular event in PAD-patients is an interesting finding that, to our knowledge, has not been reported before. A U-shaped association has been described between cystatin C and incident cognitive imparment/dementia in a longitudinal study in a population based cohort of elderly women.[175] A similar U-shaped association has also been reported between creatinine, but not cystatin C, and subclinical brain infarction in a cross-sectional study in a community based cohort of men and women > 65 years of age.[176] A third study noticed the same pattern between creatinine, but not cystatin C, and inflammatory markers (CRP and fibrinogen) in a cross-sectional study in a community based cohort of men and women with mean age of 75.[177] An interpretation of this might be that cystatin C may reflect different activities in different stages of an ongoing atherosclerotic process, such as inflammation and small vessel degeneration leading to end organ damage such as subclinical brain infarction and subsequent vascular dementia. As mentioned earlier cystatin C is an inhibitor of cathepsins,[168] with regards to that a somewhat more speculative interpretation of the U-formed shape could be that it reflects an initial depletion of cystatin C in response due to increased extracellular matrix degradation and vascular wall remodeling such as seen in atherosclerosis and abdominal aortic aneurysms.[178,179] A reciprocal upregulation may in the early phases not be fast enough to counterbalance the pathogenic mechanisms such as inflammation,[180,181] thus leading to increased events in the low tertile. In the high tertile the increased events may simply be due to the fact that cystatin C's inhibitory effect is not

efficient enough in this late stage of disease. In our fourth paper we found a connection between cystatin C and incident stroke and AMI, however this association was not U-shaped.

6.3 HERITABILITY OF THE ASSOCIATION BETWEEN CYSTATIN C & CVD

The results of our first study indicate an unambiguous connection between cystatin C and atherosclerotic cardiovascular disease. Whether this was due to a causal association, or if it merely owed up to proxy effects of impaired renal function in atherosclerotic patients is unclear. Further, we were not able to establish a predictive effect of cystatin C with regards to future cardiovascular events in an already atherosclerotically burdened cohort. This might solely be due to power issues as there are numerous studies that have proved that cystatin C is significantly associated with, and predictive of incident CVD,[171,182,183] however few studies exist in patients with manifest CVD. Thus it prompted us to further investigate the relation between cystatin C and atherosclerotic CVD in a considerably larger cohort.

Since atherosclerosis is considered a genetically complex disease we decided to investigate the importance of heritage and environment for the connection between cystatin C and prevalent as well as incident ASCVD. By using the classic twin model we estimated the heritability of both cystatin C and creatinine to just under 0.6 on average for both sexes. For cystatin C the estimate is higher than what has been found in a previous study that used an extended pedigree model, where the heritability was estimated to be 0.40 when adjusted for age and sex, and attenuated to 0.35 upon further multivariable adjustment.[184] The study by Parikh et al. differs from ours regarding methodology, size and age of the participants, therefore results may not be fully comparable. For serum creatinine, previous studies have reported heritabilities in the range of 0.00-0.64,[185-188] whereas the heritability of creatinine-based estimated GFR was 0.31-0.63. [188,189] Our heritability estimate for creatinine in is similar to that found by Jermendy et al,[186] but higher compared to what is described by Nilsson et al.[187] In accordance to our results, Nilsson et al found that the genetic influence was higher in women than in men, although under an ACE-model. In a Danish population-based twin study no heritability at all was found in men but a substantial dominance component in women.[190] However, the study cohorts in all these studies were five to tenfold smaller than ours, and the Jermendy cohort is also substantially younger, why comparisons need to be made with some caution. Further, we saw significant differences between the sexes both in terms of heritability and the proportion of the variance explained by additive and dominance genetic factors respectively. In women the broad sense heritability, here consisting of additive and dominance components contributing to the variance (Table 9), was larger than in men, which for creatinine is consistent with earlier findings.[187]

The finding of a dominance effect for cystatin C is, to our knowledge, new. In previous twin studies the ACE model has been preferred, whereas in ours the ADE model was preferred for four out of seven investigated traits. A reason for this might be the large study population which enhances power and enables detection of weaker variance components underlying the traits. Another contributing factor may be the relatively high age of the participants which might possibly lead to decreased influences from shared familial environment.
The most striking sex difference in our findings was also related to the dominance component. In men the dominance component was about the same size as the additive component whereas in women the dominance component was almost absent. Thus our findings possibly reflect an x-linked component,[191] or a gene-gene interaction specific to men. For the phenotypes in our investigations where the ACE-model was preferred (CKD-epi formula derived GFR and CVD) heritability was higher in men, but the common environment component was significantly larger in women.

The reported "chip heritability" V(g)/V(p) from the GCTA analysis in our cohort was 0.40 for cystatin C and 0.19 for creatinine. Previous studies using the GCTA method for various traits and diseases has found that the "chip heritability" tends to be in the order of ¼ to ½ of the twin-based heritability.[192] Here, we found the V(g)/V(p) to approach 65% of the twin-based sex averaged estimate for cystatin C and cystatin C based machine estimate of GFR, but only about 30% for creatinine and MDRD, which indicates that an unusually large proportion of the genetic variability for cystatin C appears to be captured by the investigated common SNP markers.

The definition of cardiovascular disease we have utilized is a commonly used endpoint in cardiovascular interventional studies and in epidemiological cohort studies. However, to our knowledge no previous study has reported heritability for this combined phenotype. The heritability of 0.39 respectively 0.20 in men and women for CVD that we report may be compared to a heritability of 0.57 in men and 0.38 in women for death by coronary heart disease reported by Zdravkovic et al.[193] When we divided CVD into its components coronary artery disease and stroke, the heritability for CAD increased to 0.48 in men and 0.30 in women. These differences may in part be explained by the fact that the Zdravkovic study only studied mortality from coronary heart disease, in which genes might be more apparent than for other manifestations. Due to a larger number of outcomes the power to assess heritability in the Zdravkovic study was better than in our cohort. Further, it may be more

difficult to delineate the role of genetic effects for composite endpoints such as CVD as risk factor patterns for the included endpoints may differ.

6.4 PREDICTIVE VALUE OF CYSTATIN C FOR INCIDENT ASCVD

The observation that common genetics appear to mediate phenotypic correlation between cystatin C and prevalent cardiovascular disease led us to further investigate the relative importance of gene and environment for the association to incident ASCVD as well. The association of cystatin C and creatinine-based eGFR to incident ASCVD was thus studied in a prospective co-twin control design. We showed that variation in cystatin C related to incident ASCVD independent of traditional risk factors and creatinine based eGFR, thus confirming earlier studies.[194-196] A novel finding was that cystatin C predicted incident stroke in MZ twins adjusted for genetic confounding.

The finding that cystatin C is superior to creatinine for prediction of incident ASCVD confirms findings from previous population-based studies,[101-103] but is for the first time observed in a twin-cohort. Besides being somewhat larger compared to the study by Svensson-Färbom the fact that our cohort consisted of twins allowed us to control for genetic confounding. We also stratified our combined endpoint into MI and stroke and were thus able to see a difference in the association of cystatin C with regards to these two endpoints. It is plausible that it primarily is a reflection of cystatin C being a better marker of early hypertensive end organ damage in different vascular beds. That is – a more sensitive marker of early GFR-reduction. In this regard cystatin C might be a marker of early vascular ageing, and as such detect subclinical manifestation of features such as small vessel degeneration, left ventricular heart load, arterial calcification and matrix remodeling and intima alterations.[105,106]

The finding that cystatin C is related to incident stroke in identical twins is a novel finding which indicates that individual specific (i.e. non-shared within pairs) environmental factors that affect cystatin C also associates to incident stroke. This is further supported by the finding that the intra-pair contact frequency and age at separation was not significantly associated with trait-level similarity in MZ twins (Table 16). Based on the findings and study design it cannot be concluded what constitutes these unique environmental factors. Previously reported environmental factors that are associated to cystatin C or mild eGFR-reduction are smoking and occupational exposure to lead and arsenic.[197-200] It could be exposure to some other external factor whose connection to cystatin C and ASCVD yet

remains to be discovered. When we adjusted for traditional risk factors such as serum cholesterol, lipids, diabetes, anti-hypertensive treatment, systolic blood pressure, smoking and decreased kidney function this association remained which indicates that the external factor is independent of them. Our findings are in agreement with recently published Mendelian randomization studies[201] which have reported negative results on the association between SNP:s related to cystatin C and incident CVD. Therefore environmental factors are indeed the most likely cause of the association between cystatin C and incident ASCVD and may also be possible to prevent if identified.

6.5 GENETIC CORRELATION OF VARIATION IN CYSTATIN C AND CREATININE WITH PREVALENT AND INCIDENT ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

We found a significant genetic correlation between levels of cystatin C and creatinine. This correlation was explained by shared genetic- and non-shared environment to approximately the same extent. Since the variations in both phenotypes reflect renal function, renal function is probably more strongly related to the covariance of the two traits than to the individual phenotypes. The observation that shared genes contributed substantially to the covariance of the two traits, and thus renal function, is an expected finding, while the small contribution of shared environment is not. The most common risk factors related to chronic kidney disease are overweight, hypertension and diabetes mellitus,[202] where genes as well as environmental factors are important in all three. In our study population very few had verified CKD and the majority of the variation of the studied phenotypes was in the normal GFR range. The contribution of these classic risk factors to the variation of the studied phenotypes in the normal range is unknown. Further, we observed genetic correlation between CVD and cystatin C despite a low phenotypic correlation between the traits, which also is a novel finding. The relation between creatinine and CVD was similar but the phenotypic correlation between these two traits was very weak. Although the variation in cystatin C only explained a small part of the variation in CVD, the covariance between the traits was entirely explained by common genes. This finding was only significant in males and may reflect that CVD was more common in men and thus a low power in the study to detect possible associations in women. It is possible that the shared genetic factor may affect all these phenotypes directly. However, the finding that cystatin C and CVD partly share common genes may also indicate a possible causal relation between cystatin C and CVD.

Previous studies have reported about genetic overlap between kidney function and ASCVD.[203] Although these overlaps were quite modest, ranging from 0.1 to 0.26 % and

associated with different stroke sub-types, they confirm earlier epidemiological studies on the matter suggesting such genetic overlaps.[204] While both creatinine and cystatin C based kidney function was used in the study by Holliday et al, creatinine was more commonly used, and since cystatin C is superior for risk prediction - further investigations on the possible genetic overlap between stroke and cystatin C was warranted. We observed a stronger association between cystatin C and incident stroke compared to incident MI. Svensson-Färbom were able to demonstrate a significant association between cystatin C and ASCVD morbidity that was not present for creatinine based eGFR until eGFR was below 45mL/min, corresponding to less than 1% of the study population. However they did not study the associations to MI and stroke separately. A plausible explanation, especially if we assume that cystatin C is a marker of small vessel disease and hypertensive end organ damage, is that in normal kidney function cystatin C captures the risk of ASCVD trough small vessel disease but when renal function declines this discrimination gets distorted and cystatin C instead captures the risk of ASCVD due to renal dysfunction.

We observed that non-shared environment might mediate intra-pair discordance regarding stroke incidence. This finding is in accordance to results from previous studies that have shown that only a small fraction of stroke variance is explained by genetic overlap with renal function estimates.[205] Further support for the small genetic overlap between these traits can be found in a study by Olden et al,[206] where SNPs associated with CKD were tested for association with CVD traits and vice versa. The result from this study concludes that there is little overlap between kidney and cardiovascular disease risk variants in the overall population and that non-genetic, i.e. environmental, factors in the causal pathway are responsible for the major part of the association between ASCVD and kidney function. Therefore, it is important to further investigate the part of the association that is related to environment and unarguable also better suited as a target for preventive measures.

The finding that cystatin C and CVD partly share common genes may indicate a possible causal relation between cystatin C and CVD. However this association is between prevalent CVD and cystatin C and hence it is not possible to draw any conclusions regarding a potential causal relationship. The association may very well be due to reversed causality, i.e. CVD in itself may have caused the elevations in cystatin C. It is also possible that the link is due to confounders that are linked to both exposure and outcome. In the longitudinal design of study IV, the time factor makes associations between exposure and outcome more straight forward as the exposure precedes the outcome. Still this is not evidence of causality, even when

adjusting for confounders, as confounding factors may be unknown or masked. However reversed causality is less likely and thus our findings may indicate that this is not the case regarding the association between cystatin C and CVD.

We do indeed present results indicating a genetic overlap between cystatin C and prevalent CVD in our studies which may indicate causal associations. This reasoning has made the relation between cystatin C and CVD suitable for Mendelian randomization studies (MR) in which firstly an instrumental variable that is related to the variance of the studied risk factor is identified (one or several SNP:s from the genome) and secondly the relation of this instrumental variable to the occurrence of the disease in question is studied.[136] MRs uses the unique properties of the genome, to investigate causality of a biomarker. The unchangeable nature of the genome and Mendel's laws of random distribution of alleles from parents to offspring at conception, means that genetic information is not influenced by disease status (reverse causality) or traditional risk factors (confounding).[130] Thus, genetic variation that controls serum concentrations of cystatin C could be used as a variable to assess the effect of elevated concentrations of cystatin C on disease risk, independent of potential confounders. This has recently been done, and findings from this recently performed Mendelian randomization (MR) study contradicts a causal relation of cystatin C with regards to coronary artery disease.[201] This has also been confirmed and extended to stroke, heart failure, diabetes and the metabolic syndrome in two other well powered MR-studies, yet only published as abstracts.[207,208] Thus a more plausible reason to the genetic overlap between cystatin C and CVD in our studies is, given these findings, that it is due to pleiotropy where the same genes affect both phenotypes independently. Further cystatin C is a marker of chronic kidney disease, which in part in itself is a form of vascular disease, such as atherosclerotic renovascular disease (ARVD),[209] which makes studies of and conclusions regarding causality utterly complex. Thus it is also possible that the seemingly independent correlation between cystatin C and PAD in our studies is due to subclinical renal failure, possibly on the basis of ARVD, that creatinine based eGFR is unable to detect.

6.6 METHODS DISCUSSION

Analysis of covariance, ANCOVA is a strong method for comparing the means of a continuous dependent variable between two or more groups with regards to levels of an independent categorical variable while controlling for a the effects of a third continuous variable. This method was successfully applied in order to analyze the separate effect of PAD

on serum cystatin C-levels and the possible interaction between PAD and renal impairment in study I.

Survival analysis using Cox-regression is a useful method to estimate the association between a risk factor and time to an event. It is most often applied in order to study the relationship between exposures and a dichotomous outcome variable such as death, onset of disease etc. Study subjects are followed from baseline until they reach either the endpoint or a censoring endpoint (for example death from other causes than the studied). The result is expressed as hazard ratios reflecting the risk of an event anytime during the study period compared to an unexposed control group. It is also possible to add multiple covariates to test for interaction, as was done in the analysis of cystatin Cs predictive value in study II and IV.

Logistic regression is a statistical model that is used to describe the association between one or several continuous or dichotomous exposure variables and one dichotomous outcome variable. This is a useful method for assessing the effect of different exposures with regards to an outcome, such as was done in study IV where monozygotic and dizygotic pairs discordant for ASCVD were compared.

6.6.1 GCTA & Twin model

Since the GCTA and twin model represent two different methods of estimating heritability, the two methods should be regarded as complementary rather than directly comparable. One of the major differences between the methods, which also likely explain the difference in the twin study estimates compared to the GCTA-estimates, is that the GCTA only estimates genetic variability captured by the SNP markers. Non-tagged polymorphism such as rarer single base-pair mutations, copy number variations and rare alleles, which will affect the estimate in the twin based model, will not contribute to the GCTA. Further, the GCTA model assumes additive genetic variance and thus only captures the narrow-sense heritability, i.e. non-additive effects such as dominance, gene-gene interactions (epistasis) and gene-environment interactions are not accounted for. There is also a possibility that the estimates of the twin-model are inflated by a violation of one or more of the general assumptions of equally shared environment, minimal gene-environment interaction, random mating or generalizability to the population, which might further widen the gap between the heritability estimates derived from the two models. The equally shared environment assumption stipulates that monozygotic and dizygotic twins are exposed to trait-relevant shared-environmental influences equally. A violation of the equal environment assumption would be

if MZ twins share more raising environment than do DZ. This would lead to an increased MZ correlation relative to DZ correlation, which would mimic genetic dominance effects and result in an overestimation of the dominance genetic effect relative to the shared environmental effect. A violation of the minimal gene-environment interactions assumption on the other hand where different genotypes responds differently to the same environment could instead lead to an inflation of the unique environment effect.

6.7 LIMITATIONS

As is the case with observational studies in general, all papers in this thesis are possibly limited by a range of different biases, ranging from selection bias due to self-selection, through misclassification of subjects regarding exposure as well as endpoint, to recall bias due to the fact that some of the data used stems from interviews and participation forms.

6.7.1 Study I & II

As pointed out earlier, our study population was quite small, which limits the power of the studies. Further the study population consisted of men only which limits the findings with regard to gender, although it increases the homogeneity of the study. The case–control design of study I also precludes us from drawing conclusions about causal mechanisms. Another limitation of our study is that we used the MDRD equation to calculate GFR instead of directly measuring for example iohexol clearance. Morbidity and mortality data were acquired from the Swedish National Board of Health and Welfare, and these data are based on hospital records from treating physicians, which might be associated with ascertainment biases. To limit the risk of misdiagnosis, we have reviewed the hospital records to validate the diagnosis of hospitalization and causes of mortality.

6.7.2 Study III & IV

A well-known limitation of twin studies is that it is not possible to incorporate the effect of shared environment and dominance genetics simultaneous, therefore both factors may co-exist but not be detectable in the same model. Similarly the estimated effect from additive/dominance genetic effects may potentially arise from epistasis or gene-environment interactions, why all results on additive and dominance genetics could be biased. Moreover, the fact that the classic twin design relies on four general assumptions makes it vulnerable, especially with regards to the assumption of equal environment influences between MZ and DZ which may result in an overestimation of the genetic effect

and an underestimation of the shared environmental effect. Still, in order for an equal environments assumption violation to be a major problem there must be an association between degree of shared environment and trait similarity. Further, variance components estimates are study population-specific, meaning that they might not be representative for other populations or ethnic groups. Since both genetic and environmental factors may be explicit to the studied population it may be these factors that drive the difference between studies. Since our cohort consists mainly of elderly individuals the common environment factor may be less prominent than in a younger population, and thus make generalizability lower.

A limitation of the genome wide complex trait analysis is that it can only detect the additive effects of the common SNPs (i.e. with allele frequencies greater than 1%) that are incorporated in the DNA microarrays used in genome-wide association studies and not non-additive effects such as gene-gene or gene-environment interactions. [210] Further, GCTA estimates are the result of using only SNPs but there are numerous other kinds of prevalent genetic polymorphisms, such as copy number variations, multiple copies of segments of genes, whole genes, and even whole chromosomes and thus it might overlook a lot of important genetic variation. A third limitation that may influence the accuracy of the GCTA is the risk of confounding due to population stratification. This type of bias is due to the fact that there is a chance that genetically related people tend to be geographically proximal.[211] Population stratification is thought to be avoided by the use of principal components,[212] but there is growing evidence that this technique might not be sufficient.[213] Just as in the preceding studies, studies III and IV suffers from limitations due to possible inaccuracy of morbidity and mortality data. Further, they are also limited by having to use different estimations of GFR and not inulin or iohexol clearance.

6.8 CLINICAL IMPLICATIONS

Cystatin C is an easy to measure, commonly available, serum analysis already widely used in the clinic. Its association to atherosclerotic cardiovascular disease, whether it is causal or due to the proxy measure of renal dysfunction, is unambiguous. The results presented in this thesis expand the knowledge on the relation between cystatin C and CVD, specifically as a marker for environmental exposure associated to incident CVD. We argue that our results support the use of cystatin C as an important biomarker sensitive for incident CVD. Thus it could be of value to expand its usage beyond renal medicine and include it as a tool in the arsenal for cardiovascular risk stratification.

A valid question is of course how much value will be added to risk stratification by including cystatin C on top of classic CVD risk markers. Two longitudinal studies have shown an association of an increase in cystatin C, adjusted for the classic Framingham risk factors, with an increased risk of CVD. [183,214] Still, a statistically significant correlation is not the same as a reclassification in terms of risk and thus may not lead to change of preventive or therapeutic management. Of the two studies mentioned above only the latter one found a statistically significant improvement of risk stratification in elderly men without cardiovascular disease when adding cystatin C in a multi-marker approach together with NT-proBNP, CRP and troponin I to the Framingham risk factors.

As mentioned in the background of this thesis the majority of events will occur in individuals at moderate risk and thus the effect of incorporating cystatin C in this group may be marginal. Nevertheless, certain individuals in high risk populations may benefit from a better stratification as it could lead to targeted interventions that reduces their absolute risk. However the main focus of the papers in this thesis has not been to evaluate how big a plausible contribution of cystatin C as a risk marker added to present risk scoring systems would be.

6.9 FUTURE PERSPECTIVES

Through our studies we have been able to detect a solid association between cystatin C and atherosclerotic disease. Since large studies do not support a causal relation between cystatin C and CVD a continued focus on establishing evidence of causality, with the prospect of future interventions targeted directly at cystatin C, is probably of less interest.
We observed that a unique environmental factor that associate to, and possibly mediate, intra-pair discordance regarding stroke incidence was mirrored by difference in levels of cystatin C at baseline. This is, in accordance with results from previous studies that have shown that only a small fraction of stroke variance is explained by genetic overlap with renal function estimates. Thus an important future focus is to investigate the part of the association between cystatin C and ASCVD that is related to environmental factors and which is better suited as a target for preventive measures.

Finally, studies regarding the usage of cystatin C as a predictive biomarker of different forms of cardiovascular disease, in different populations, are needed in order to further investigate a possible clinical use. Specifically studies that can show if the biomarker can improve

reclassification to relevant risk categories are warranted as are studies that provide cost – benefit measures such as numbers needed to screen. Further the observed U-formed relation need additional investigation.

7 CONCLUSIONS

7.1 STUDY I

Cystatin C concentration, adjusted for differences in eGFR, IL-6 and CRP was higher in PAD-patients compared to controls. Thus cystatin C may be an independent marker of atherosclerotic disease apart from its relation to kidney function.

7.2 STUDY II

Cystatin C could not predict CV events and did not improve discrimination or reclassification in patients with peripheral arterial disease although an interesting finding was a U-formed relation between cystatin C and CV events. An interpretation of this might be that cystatin C may reflect different activities in different stages of an ongoing atherosclerotic process.

7.3 STUDY III

The heritability of Cystatin C was 0.60 (0.56-0.63) and the heritability of creatinine was 0.59 (0.56-0.62) which is higher compared to previous studies. The GCTA analysis provided independent evidence for the significant heritability indicated by the twin-model for all phenotypes. Cystatin C was weakly correlated to prevalent CVD, although more strongly than creatinine, and the covariation between cystatin C and CVD in males was explained by additive genetic components indicating that cystatin C and CVD share genetic influences.

7.4 STUDY IV

Variation in cystatin C was associated with incident atherosclerotic cardiovascular disease, independent of traditional risk factors, with a stronger association to stroke which remained after adjustment for genetic confounding. The finding that cystatin C is related to incident stroke in disease-discordant identical twins is novel and indicates that individual specific environmental factors that affect cystatin C also associates to stroke risk.

7.5 OVERALL CONCLUSION

Cystatin C is associated to atherosclerotic disease. The covariation between cystatin C and CVD in males indicates that cystatin C and CVD share genetic influences. Variation in cystatin C is associated with incident myocardial infarction and stroke independent of

traditional risk factors, with a stronger association to stroke. The finding that cystatin C is related to incident stroke in disease-discordant identical twins indicates that individual specific environmental factors are important. One possible explanation is that cystatin C may be a sensitive marker of early hypertensive end organ damage. It could be of value to expand the usage of cystatin C beyond renal medicine and include it as a tool in the arsenal for cardiovascular risk stratification. However, further research is needed.

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